DEVELOPMENT OF HYBRID YEAST STRAINS FOR THE PRODUCTION OF BIOETHANOL FROM LIGNOCELLULOSIC BIOMASS

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CERTIFICATE

This is to certify that the thesis entitled **"Development of hybrid yeast strains for the production of bioethanol from lignocellulosic biomass"** submitted by **Rajni Kumari** of National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance. The candidate has fulfilled all prescribed requirements for Ph.D. dissertation.

To the best of my knowledge, the matter embodied in the thesis is based on candidate's own work and the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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ABSTRACT

In recent years, lignocellulosic biomass is considered as a potential feedstock for the production of bioethanol because of its abundant availability, low cost and renewable sources. However, the lack of efficient microorganism to ferment pentose and hexose sugars released from lignocellulosic materials is one of the main factors limiting the utilization of lignocellulose for actual bioethanol production. Further, commercially during fermentation, yeast cells are subjected to multiple stresses that affect the bioethanol production. Therefore, the main focus of the present research involves the development of hybrid yeast strains that are capable of fermenting hexose and pentose sugar components of lignocellulosic biomass even under the stress conditions. Various hybrid yeast strains were prepared by the fusion of protoplasts of *S. cerevisiae* and a variety of xylose fermenting yeasts such as *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae*. Among the various fusants, fusant RPR39 comprising of *S. cerevisiae* and *P. tannophilus* was found to be the most efficient strain giving maximum ethanol concentration of 76.8 gL^{-1}, ethanol productivity of 1.06 $gL^{-1}h^{-1}$ and ethanol yield of 0.458 gg^{-1} by fermentation of glucose-xylose mixture. The fusant RPR39 was further subjected to sequential mutagenesis for improvement of its various important properties such as stability and stress tolerance using various mutagenic agents. The mutants were evaluated for their tolerance to ethanol, temperature, fermentation inhibitors and stability. Among these mutants, mutant RPRT90 exhibited high ethanol tolerance, inhibitor tolerance and reasonably good thermotolerance. The mutant RPRT90 showed improved ethanol production (73.6 gL^{-1}) from glucose-xylose mixture with higher ethanol yield (0.461 gg^{-1}), productivity (1.05 $gL^{-1}h^{-1}$) and sugar conversion (86.2 %) compared to fusant RPR39. The strain has shown its efficiency towards ethanol production under various stress conditions during fermentation of glucose-xylose mixture (3:1 ratio). Under the combined effect of thermal (39^{o}C) and inhibitor stress (0.25 gL^{-1} vanillin, 0.5 gL^{-1} furfural and 4 gL^{-1} acetic acid), the mutant produced ethanol with a yield of 0.379 gg^{-1}, while under combined effect of ethanol (5 %, v/v) and inhibitor stress, the ethanol yield was 0.431 gg^{-1}. Even, under the synergistic effect of thermal (39^{o}C), ethanol (5%, v/v) and inhibitor stress, the strain has shown to be active achieving good ethanol yield (0.3 gg^{-1}) productivity (0.59 $gL^{-1}h^{-1}$). Further, the developed yeast strain has been successfully applied to produce bioethanol from the locally available lignocellulosic biomass, *Ipomoea carnea* and *Lantana camara*. Under the optimum conditions of biomass conversion steps, the mutant hybrid strain has shown

encouraging results in fermenting the mixed hydrolysates obtained from both *I. carnea* and *L. camara*. The fermentation of *I. carnea* mixed hydrolysate containing the detoxified acid hydrolysate (18.69 gL^{-1} sugar) and enzymatically hydrolysed cellulosic hydrolysate (48.10 gL^{-1} sugars) produced 27.2 gL^{-1} ethanol, with ethanol yield and productivity of 0.456 gg^{-1} and 0.971 $gL^{-1}h^{-1}$. The ethanol produced from the mixture of undetoxified acid hydrolysate and enzymatic hydrolysate was found as 23.01 gL^{-1}, with the ethanol yield and productivity of 0.415 gg^{-1} and 0.821 $gL^{-1}h^{-1}$. Thus, it has been established that the developed strain RPRT90 is an efficient strain that may pave the way to produce bioethanol from lignocellulosic biomass economically at an industrial scale. Furthermore, a comparable ethanol yield and productivity of 0.434 gg^{-1} and 0.412 gg^{-1} were achieved with detoxified and undetoxified hydrolysates derived from *L. camara* biomass by fermentation using RPRT90 strain.

Keywords:

Bioethanol, Lignocellulosic biomass, *Saccharomyces cerevisiae*, Xylose-fermenting yeast, Protoplast fusion, Mutagenesis, Mixed hydrolysate, *Ipomoea carnea*, *Lantana camara*, Fermentation inhibitors.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ANOVA	Analysis of variance
BSA	Bovine serum albumin
DNS	Di-nitro salicylic acid
EMS	Ethyl methyl sulfonate
FACS	Fluorescent activated cell sorter
FDA	Fluorescein di-acetate
FITC	Fluorescein iso-thio cyanate
FTIR	Fourier transform Infrared spectroscopy
HPLC	High pressure liquid chromatography
MNNG	N-methyl N-nitro N-nitrosoguanidine
NCIM	National collection of industrial microorganisms
PEG	Polyethylene glycol
PI	Propidium iodide
R6G	Rhodamine 6G
RAPD	Random amplified polymorphic DNA
RSM	Response surface methodology
SEM	Scanning electron microscope
TCA	Tricarboxylic acid
XRD	X-ray Diffraction
β-NAD	Beta-nicotine amide di-nucleotide
CSF	Combined severity factor
BBD	Box-Behnken Design

Chapter 1

INTRODUCTION

1.1 Background

The steep rise in global warming, depletion of oil reserves and exorbitant rates of gasoline has drawn attention in recent years towards the production of bioethanol as an alternative source of transportation fuel. To implement the stringent environmental protection law, there is growing demand of using bioethanol as an alternative fuel in many countries. However, to promote bioethanol utilization and its production at industrial scale, the choice of feedstock is an important issue. At present, bioethanol is produced commercially from a variety of food crops, the most important of which are sugarcane and corn. The use of these foodstuffs leads to an undesirable competition between food security and bioethanol production. Moreover, bioethanol production from these edible crops is not cost effective as compared to the petroleum based gasoline. Therefore, in recent years, much research interest has been generated to produce bioethanol from a variety of lignocellulosic biomass because of their abundant availability over earth's surface, low cost and potentiality to produce clean fuel [1]. It has been reported that the exploitation of these resources may provide a sustainable energy supply at a local, regional and national level [2]. However, the conversion of this biomass to bioethanol is much difficult due to complexity of its characteristics. Lignocellulosic biomass is mainly comprised of cellulose, hemicellulose and lignin polymers. The cellulose and hemicellulose component of the biomass are the potential source of sugar substrates that can be fermented to ethanol. Furthermore, among the pentose and hexose sugars present in lignocellulosic biomass, glucose and xylose are the most abundant sugars. However, it has been reported that the lack of efficient microorganism to ferment a variety of sugars released by the hydrolysis of lignocellulosic materials is one of the major factors limiting the complete utilization of lignocellulose for bioethanol production [3]. Therefore, the major challenge lies in this area is the development of an efficient and stable microbial strain that has the ability to co-ferment pentose and hexose sugar components present in the lignocellulosic biomass. Protoplast fusion is an important and powerful technique that has been used to combine genes from different microorganisms to develop hybrid strains with desired industrial properties [4]. Therefore, recent research has been focused on the development of a hybrid strain from the most industrially important yeast strain, *S. cerevisiae* and potential xylose fermenting yeasts by protoplast fusion. As a result, different hybrid strains have been developed by researchers [4, 5]. However, the study on the bioethanol production from lignocellulosic substrates using these fusant strains is inadequate. Therefore, a systematic research effort is urgently required

for the development and utilization of these hybrid strains for actual bioethanol production from lignocellulosic substrates at the cost more competitive with petroleum based gasoline without compromising with food grains.

1.2 Bioethanol as a sustainable transportation fuel

The expansion of energy sector is essential for accelerating the economic growth of a nation and sustenance of a modern economy. Further, with the development of energy sector, it is equally important that all issues concerning environment protection and enrichment are also tackled sincerely. It is obvious that the future economic growth crucially depends on the long-term availability of energy from sources that are affordable, accessible and environmental friendly. Thus, a nation needs to adopt clean and efficient technologies to reduce its green house gas emissions. The emphasis is on reducing pollution and helping to satisfy the Kyoto protocol, established in 1997, by limiting the global net emission of carbon dioxide (CO_2). The limited availability of fossil fuels and the growing awareness of the detrimental environmental consequences resulting from greenhouse gas emissions have reinforced the importance of alternative energy resource in developed and developing countries. In this context, bioethanol can be a potential alternative energy source. Bioethanol is a renewable and clean fuel as unlike petroleum it emits 35% less carbon monoxide, 42% less nitrogen oxides, 79% less carbon dioxide, 43% less hydrocarbons and 39% less particulate matter [1]. Furthermore, bioethanol can be produced in short duration of time. It is also reported that as ethanol is derived from plant matter, its use as a fuel does not contribute to the net accumulation of CO_2 in atmosphere [6].

Ethanol or ethyl alcohol (C_2H_5OH) is a clear colourless liquid; it is biodegradable, low in toxicity and causes little environmental pollution. It has a higher octane number, broader flammability limits, higher heats of vaporization and higher compression ratio than gasoline which lead to high efficiency in an internal combustion engine [2]. Blending ethanol with gasoline can also oxygenate the fuel mixture so that it burns more completely and reduces polluting emissions. Ethanol fuel blends are widely sold in the many countries and the most common blends are E5 (5% ethanol and 95% petrol) and E85.

1.3 Lignocellulosic biomass and its chemistry

Any raw materials containing sugars, or materials that can be transformed into sugars, can be used as fermentation substrates for bioethanol production. Presently bioethanol is produced from corn in USA or sugarcane in Brazil. However, to enable a more substantial increase in world-wide ethanol production capacity, lignocellulosic substrates need to be used. Lignocellulosic biomass are renewable, inexpensive, environmental benign and most importantly, it does not interfere with the food security. Moreover, second generation production methods that can convert lignocellulosic plant matter into ethanol would allow more biomass to be converted to ethanol, either by using the conventional crops or faster growing crops.

Lignocellulosic feedstocks can be categorised into four main groups: dedicated energy crops, agricultural residues, wood residues and municipal paper waste. All of these feedstocks are abundantly available over earth's surface and ethanol production from these feedstocks could also solve the problem of their disposal. Furthermore, lignocellulosic biomass is a carbon neutral source of energy as the combustion of lignocellulosic ethanol produces no net carbon dioxide into atmosphere. Fermentation of these residues to ethanol is an attractive way to supplement the fossil fuels.

The major types of biomass for ethanol production recognized are monoculture crops grown on fertile soils (such as sugarcane, corn, soya beans, oilseed, switch grass, willow, and hybrid poplar), waste biomass (such as straw, corn stover, and waste wood), and municipal solid waste (such as processed paper and newspaper). Weeds like *Ipomoea carnea, Eicchornia crassipes, Lantana camara, Prosopis juliflora, Saccharum spontaneum, Typha latifolia, Crofton, Chromolaena odorata,* etc., are another type of biomass which are promising and cheaper feedstocks for fuel ethanol production. These weedy cellulosic substrates do not require additional economic input as they grow on agriculturally degraded land or water bodies. Thus, weed biomass can be a potential feedstock for bioethanol production.

Chemistry of lignocellulose

The lignocellulosic biomass consists of five major components: cellulose, hemicellulose, lignin, protein and inorganic matter. The major constituents include structural and non-structural carbohydrates (cellulose and hemicellulose) and lignin. The cellulose and hemicellulose are the most essential components for ethanol production as they are the polymers of various sugar monomeric units. The concentration of each class of compound varies depending upon the species, type of plant tissue, stage of growth, and growing conditions. Due to carbohydrate structure, biomass is highly oxygenated with respect to conventional fossil fuels, including hydrocarbon liquids and coals. The principal constituent of biomass is carbon (30–60 wt% of dry matter) and other is oxygen (30–40 wt% of dry matter). The third major constituent is hydrogen, comprising typically of five to six per cent dry matter. Nitrogen, sulphur, and chlorine usually comprise less than one percent dry matter.

Cellulose is a linear homopolysaccharide composed of elementary links of anhydro-D-glucose and represents a poly-l, 4-D-glucopyranosyl-D-glucopyranose. The β-D-glucopyranose units linked together by (1-4)-glycosidic bonds. The cellulose molecules are linear; the β-D-glucopyranose chain units are in a chair conformation and the substituents HO-2, HO-3, and CH_2-OH are oriented equatorially [7]. Glucose anhydride, which is formed via removal of water from each glucose molecule, is polymerized into long cellulose chains that contain 5000–10,000 glucose units. The basic repeating unit of the cellulose polymer consists of two glucose anhydride units, called a cellobiose unit [7]. Cellulose is the main component of the cellular walls of higher plants. The structure of a linear polymer of cellulose is shown in figure 1.

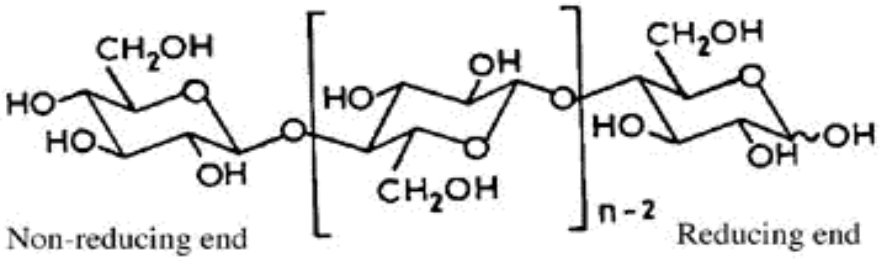


Fig. 1: The structure of cellulose polymer

Hemicellulose is a polysaccharide present in plant cell wall together with cellulose and lignin. It includes both five and six carbon monosaccharide units, with the main monomeric units being xylose, glucose, mannose, galactose, arabinose and uronic acids. Hemicelluloses are the linking material between cellulose and lignin. Hemicelluloses are highly branched hetero-polymers having degree of polymerisation within 100 to 200, comprising predominantly xylose, plus glucose, mannose, galactose and arabinose, as well as different sorts of uronic acids [7]. The structure of a linear polymer of hemicellulose is shown in figure 2.

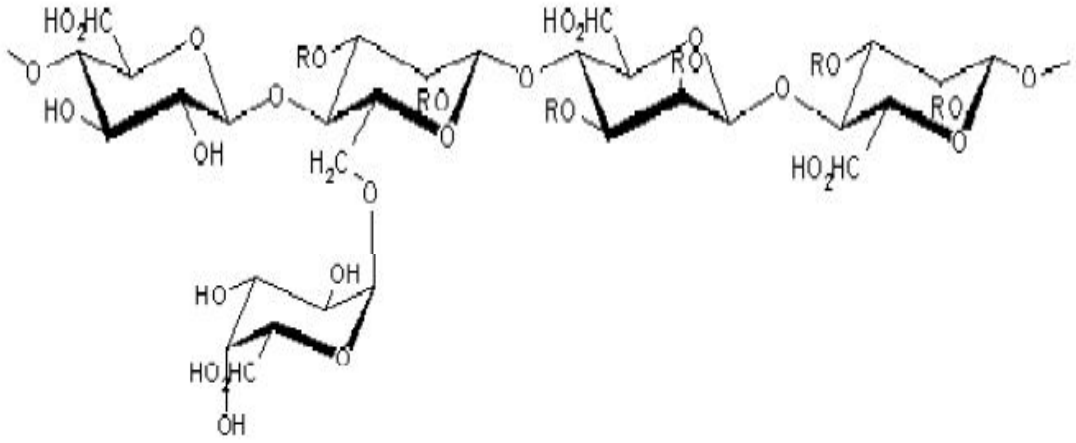


Fig. 2: The structure of hemicellulose polymer

Lignin is a three-dimensional polyphenolic network built up of dimethoxylated (syringyl), mono-methoxylated (guaiacyl) and non-methoxylated (p-hydroxyphenil) phenylpropanoid units, derived from the corresponding p-hydroxycinnamyl alcohols, which give rise to a variety of sub-units including different ether and C-C bonds [7]. The structure of lignin is complex (fig 3).

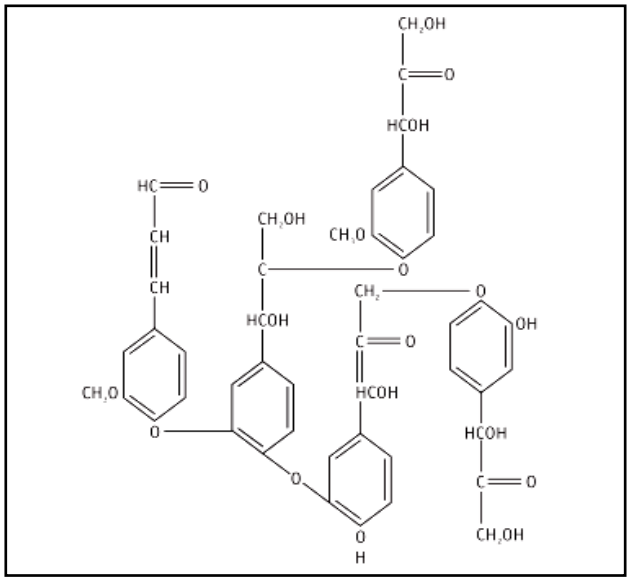


Fig. 3: Structure of a section of a lignin monomer

It deposits in an amorphous state surrounding the cellulose fibres and is bound to the cellulose directly by ether bonds. The complexity of lignin resists attack by most microorganisms (aerobic and anaerobic) and it is not considered fermentable or digestible.

1.4 Biomass conversion

The conversion of lignocellulosics to bioethanol consists of three major steps: pretreatment, hydrolysis and fermentation. In order to produce sugars from the biomass, to reduce the size of the feedstock and to open up the plant structure, the biomass is pre-treated with acids or enzymes. The cellulose and the hemicellulose portions are broken down by enzymes or dilute acids into sugar monomers that are then fermented into ethanol. The major steps involved in bioethanol production are represented as flowchart in Fig 4.

Pretreatment is required to alter the macroscopic, microscopic size and sub-microscopic structures of biomass as well as its chemical composition so that hydrolysis of carbohydrate

fraction to monomeric sugars can be achieved more rapidly and with greater yields. The main factors governing the lignocelluloses breakdown to fermentable monosaccharides are the reduction in cellulose crystallinity and the removal of lignin. An efficient pretreatment must meet the following requirements: (i) improve the hydrolysis of cellulose and hemicellulose to subsequently form sugars (ii) avoid degradation or loss of carbohydrate, (iii) avoid formation of by-products that are inhibitory to hydrolysis and fermentation processes, and (iv) be cost effective.

Pretreatment is followed by the breakdown of carbohydrate polymers to free sugar monomers. The process is termed as hydrolysis as the process involves the addition of one water molecule for every glycosidic bond broken.

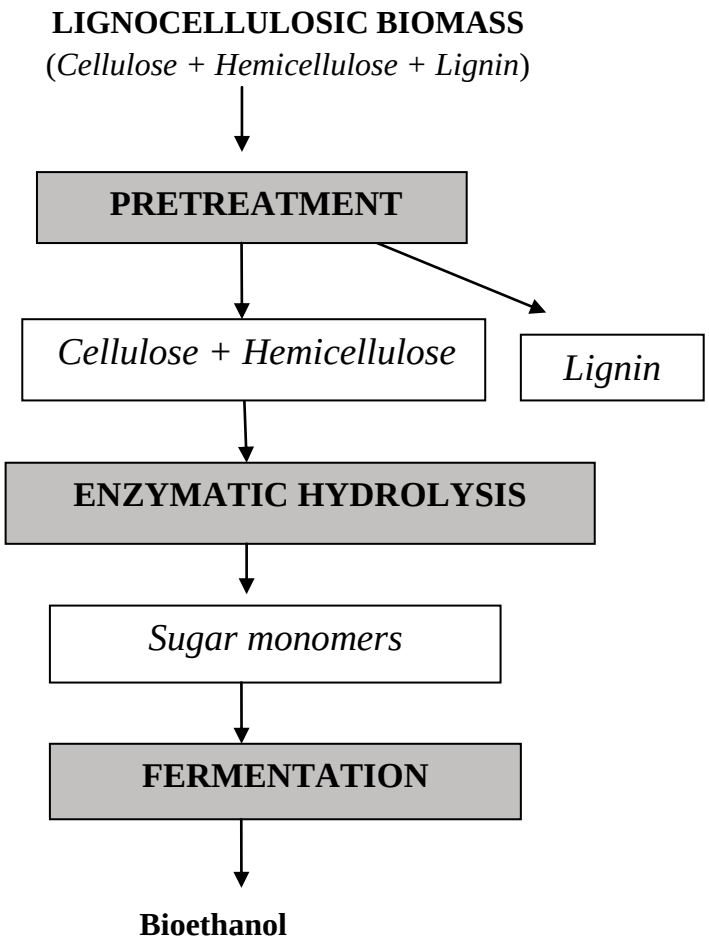


Fig. 4: Steps of bioethanol production

The most commonly used hydrolysis methods are acid and enzymatic hydrolysis. Enzymatic hydrolysis is advantageous over acid hydrolysis as it offers higher yields, minimal by-product formation, low energy requirements, mild operating conditions, and low chemical disposal costs. The cellulase enzymes employed for cellulose hydrolysis to glucose are mainly categorized in three groups: endo-glucanases, exo-glucanases and beta-glucosidases. Once the carbohydrate polymers are hydrolysed into free sugar monomers they can be fermented to ethanol using various ethanologenic microorganisms. Yeast is the most commonly used organism for ethanol fermentation, however few species of bacteria like *Zymomonas mobilis* are also used.

1.5 Yeast strains for ethanol fermentation

The biggest obstacle in the fermentation of lignocellulosic hydrolysates is the lack of efficient microorganism for fermenting both hexose and pentose sugars present in biomass. Although *S. cerevisiae* is the most commonly used for the fermentation of hexose sugars present in lignocellulosic biomass but it does not use xylose as a carbon source, which is the second major sugar component of biomass. Therefore, attention has been focused on the use of *Pachysolen tannophilus, Candida shehatae* and *Pichia stipitis,* which are the best native xylose-fermenting yeasts known [8]. Many improvements have been made in the genetic engineering of yeasts for the fermentation of xylose and arabinose to ethanol.

Development of hybrid yeast strains

The various techniques used for the genomic manipulation of yeasts include protoplast fusion, mutagenesis and recombinant DNA technology. Among these, protoplast fusion is a simple and widely used method to improve the desired fermentative properties of yeasts. It is a physical phenomenon in which the two or more protoplasts come in contact and adhere to one another resulting in the transfer of relatively large segments of genomic DNA [9]. The process of protoplast fusion involves the breakdown of cell wall, release of viable protoplasts, regeneration of protoplasts etc. Fusion of the two protoplasts may result in hybridization of characteristics of the parental strains and the progeny will express characteristics of either of parental strains or a hybrid expression may occur [9].

Mutation is defined as the permanent alteration of one or more nucleotides at a specific site along DNA strand [10]. Mutations can be spontaneous or induced, and induced mutations can be brought either by random or site directed mutagenesis. Random mutagenesis, linked with efficient selection methods is a very powerful tool to generate new strains with desired characteristics. Induced mutagenesis using physical and chemical mutagens seems to be a simple and rational approach for yeast strain improvement [10]. Researchers have reported the improvement of the yeast strains using ultraviolet radiation and chemical mutagens. UV, ethyl methane sulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment has been found to influence the different metabolic activities in yeasts. The use of multiple mutagenesis cycles to introduce multiple mutagens may be advantageous for development of new traits [11].

1.6 Organisation of thesis

The work embodied in this thesis has been presented in the following six chapters-

Chapter I presents a brief introduction emphasizing on the bioethanol, lignocellulosic biomass and yeast strains used for conversion of sugars present in biomass. The problem with the existing strains and the need for the development of hybrid yeast strain has been highlighted. A brief introduction on the various techniques involved in the development of hybrid yeast strains and significance of present study has also been included.

Chapter II presents an extensive literature survey on history of bioethanol, ethanol producing native and hybrid yeast strains, lignocellulosic biomass and its conversion process.

Chapter III includes the aims and scope of the present work.

Chapter IV describes the materials and detailed experimental procedure to carry out the various stages of present research work including **i)** Development of hybrid yeast strains by protoplast fusion, **ii)** Improvement of developed hybrid strain by mutagenesis **iii)** Bioethanol production from lignocellulosic biomass-pretreatment, enzymatic hydrolysis and fermentation and **iv)** Analytical methods

Chapter V deals with the results and discussion on the experimental work. The results and discussions section is divided into three phases. The development of fusants hybrid and selection of the most efficient hybrid strain are included in the first phase. The second phase

involves the mutagenesis of the developed fusant with the aim to improve different industrially important properties like stress to multiple tolerances. The results of various biomass conversion steps such as dilute acid pretreatment, enzymatic hydrolysis and fermentation of different lignocellulosic biomass has been presented in the third phase of this chapter.

Chapter VI includes a brief summary and conclusion of the whole investigation.

Chapter 2

LITERATURE REVIEW

Fossil fuels account for over 80.3% of the primary energy consumed in the world, and more than 55% of it is used in the transport sector. Further, transport sector is also responsible for adding high amount of pollutants in the atmosphere. The simplest way to increase the availability of fuel in transport sector and simultaneously reduce the pollution is the replacement of fossil fuels like gasoline and diesel with biofuels [1]. Bioethanol is a potential alternative biofuel for transport sector as it is produced from renewable resources like plant biomass.

2.1 Fuel ethanol- a historical perspective

Ethanol has been used as transportation fuel since 1897 when internal combustion engine (ICE) was invented by Nikolas Otto [12]. The first report on use of ethanol as fuel in United States was in 1908, when Henry Ford designed the Ford Model T to run on either gasoline or pure alcohol. He also quoted ethanol as 'the fuel of the future'. Though the first ethanol-gasoline blend was used in 1920s, ethanol was considered as an alternative fuel only after oil crisis in 1970s and since 1980s ethanol was established as the possible alternative fuel in many countries [13]. Later on, the disruptions of oil supply and environmental concern over the use of gasoline has renewed interest in the use of ethanol. Presently, ethanol blends is being used in most of the countries due to laws and recommendations in Alternative Motor Fuels Act (1988), Clean air act (1990), Energy policy Act (2005) and the Renewable fuel standard program [14].

The Kyoto protocol (1997) of the United Nations Framework Convention on Climate Change (UNFCCC), proposed that the developed countries should promote the use of renewable energy worldwide and decrease the net emission of CO_2 to atmosphere [14,15]. In this context, the bioethanol has been considered as a clean fuel to be used to meet the energy requirements in the transportation sector. The carbon dioxide emission during its combustion in engines is balanced by the CO_2 amount sequestered from the atmosphere during the growth of the plants. This results in a closed carbon cycle [2]. Moreover, the use of blends of ethanol and gasoline with higher octane rating results in a reduced engine heat and wears thus increasing the engines performance [16]. Also, a diversification of fuel sources may reduce the dependence on gasoline and increase stability of supply.

Presently ethanol is used in Brazil, United States and some European countries as a transportation fuel on a large scale. It is expected to be the most dominant renewable transportation fuel in coming two decades. Currently, the top five producers of ethanol are United States, Brazil, China, France and India. Among these, Brazil and United States together account for 60% of world ethanol production. The ethanol production in Brazil mainly utilizes the sugar cane as substrate and United States relies on corn. However, the utilisation of these substrates as animal feed and human needs limits their use for bioethanol. Therefore, in the recent years more emphasis has been given for the exploitation of some abundant, cheap and non-edible feedstocks for ethanol production. Lignocellulosic feedstocks are presently the major focused materials in regard to the cheap and abundant feedstock for bioethanol production commercially.

2.2 Lignocellulosic biomass

Different regions of the world have excess lignocellulosic biomass such as agricultural or forest waste products with high potentials for conversion into ethanol. For example, eucalyptus is abundant in Portugal, pine in Chile, corn stover in USA and Brazil has surplus sugarcane. Lignocellulosic biomass can be a potential feedstock for large scale production of bioethanol. Being abundantly available and outside the human food chain makes lignocellulosic materials relatively inexpensive feedstocks for ethanol production. Therefore, most of the countries are looking for the ways to utilize their natural resources for the production of fuel ethanol.

The lignocellulosic biomass is mainly comprised of carbohydrate polymers (cellulose and hemicellulose) and phenolic polymers (lignin) [7, 17]. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds and covalent bonds. Cellulose is a homoploymer of glucose, while hemicellulose is a heteroploymer comprising of pentose sugars like xylose and arabinose and hexose sugars like glucose, mannose and galactose. The cellulose and hemicellulose component of biomass typically make up two-third of cell wall dry matter and they are the most abundant source of sugar substrate that can be fermented to produce ethanol [17]. However, the relative proportion of individual sugars varies from one raw material to other. The hemicellulose fraction of hardwoods and agricultural raw materials are rich in pentose sugars, while softwood hemicellulose contains minor fractions of the pentose sugars

[18]. The feasibility of a lignocellulosic feedstock to be used as substrate for ethanol production is indirectly related to cellulose, hemicellulose and individual sugar concentration in feedstock.

The biological process of the converting lignocellulosic biomass to fuel ethanol involves the hydrolysis of carbohydrates polymers to sugar monomers and their fermentation using ethanologenic microorganisms like yeasts.

2.3 Ethanol fermenting yeast strains

Yeast is the most commonly used microorganism for ethanol production by fermentation process. Among several genus of yeast that can be used for ethanol production, *Saccharomyces* is most popular, because of its high efficiency in ethanol production, fast growth rates and unique tolerance to environmental stresses such as high ethanol concentration, toxic fermentation inhibitors and low oxygen levels [5]. Though *S.s cerevisiae* is considered as the most effective organism for the fermentation of cellulosic sugars but the strain cannot ferment hemicellulose derived pentose sugars, which is the second most abundant fermentable sugar component of lignocellulosics [5].

Karczewska et al. first reported that xylose can be converted to ethanol by yeasts [19]. Since then several laboratories have been trying to find out yeast strains that can ferment xylose to ethanol. Later, in 1980s it was reported that few yeast strains like *Pachysolen tannophilus* and *Candida tropicalis* has the ability to ferment xylose [20]. These yeast strains were called as xylose fermenting yeasts. Within few years around 22 yeast strains have been screened intensively to produce ethanol from D-xylose. However, only six of these (*Brettanomyces naardenensis, Candida shehatae, Candida tenuis, P. tannophilus, Pichia segobiensis, and Pichia stipitis*) produce significant amounts of ethanol and the attention was mainly focused on *C. shehatae, P. tannophilus, and P. stipitis* which are seemed to be the most efficient xylose fermenting yeasts [8].

The studies on the ethanol fermentation by xylose fermenting yeasts revealed that these strains produced ethanol with low yield and low rate of fermentation. Further, they have difficulty to optimize physicochemical parameters of the process and less tolerance to inhibitors generated during pre-treatment and hydrolysis of the lignocellulosic material

compared to common glucose fermenting yeast [21]. Thereafter, the research on ethanol fermenting yeasts was focused on development of hybrid microbial strains with the aim to have desirable properties for the fermentation of both hexoses and pentoses.

2.4 Development of hybrid yeast strains

Various methods have been the choice of researchers to develop hybrid yeast strains like protoplast fusion [4], adaptation [22], random and site directed mutagenesis [11, 23], recombinant DNA technology [5] etc. Among these protoplast fusion and random mutagenesis are most commonly used as these methods are simple, cheap and effective.

Protoplast fusion

Protoplast fusion is an effective technique for the improvement of yeast strains. It is an important tool for gene manipulation as it breaks down the barriers to genetic exchange imposed by conventional mating systems and also allows the transfer of relatively large segments of genomic DNA [9]. The first report on protoplast fusion in yeasts was on the fusion of respiratory-sufficient and deficient strains of *S. cerevisiae.* The fusants obtained were with respiratory competence and nuclear complementation [24]. Thereafter several studies confirmed the occurrence of plasmogamy and karyogamy in hybrids [25, 26].

The detailed study of protoplast fusion revealed that normally isolated protoplasts carries negative charge (-10 mV to -30 mV) around the outside of the plasma membrane due to intramembranous phosphate groups and two protoplast repel each other due to same charge. Therefore, the fusion of two protoplasts needs a fusion inducing chemical that reduces the electro-negativity of isolated protoplasts and facilitates their fusion [27]. There are several explanations which have been put forward by a number of researchers to explain the mechanism of protoplast fusion. According to one of the theory, the adherence of the protoplasts is followed by an induction phase which involves the change in the electrostatic potential of the membrane resulting in the fusion of adhered protoplasts [28]. The surface potential of the fused protoplasts returns to its former state after fusion. The external fusogens are reported to cause disturbance in the intra-membranous proteins and glycoproteins of the

adhered protoplasts. This increases membrane fluidity and creates a region where lipid molecule intermix, allowing coalescence of adjacent membranes. The addition of Ca ++ ions causes reduction in the zeta potential of plasma membrane and under this situation protoplasts are fused [28]. The high molecular weight polymer (1000-6000) of PEG acts as a molecular bridges connecting the protoplasts. On elution of the PEG, the surface potential are disturbed, leading to intramembrane contact and subsequent fusion. Besides this, strong affinity of PEG for water may cause local dehydration of the membrane and increase fluidity, thus inducing fusion. Protoplast fusion takes place when the molecular distance between the protoplasts is 10Å or less. This indicates that protoplast fusion is highly a traumatic event [27].

The protoplast fusion of glucose and xylose fermenting yeasts was first reported by Wang et al. [29]. They reported the fusion of *P. tannophilus* NRRL Y-2460 and *S. cerevisiae* to produce fusants capable of producing and tolerating 10% (v/v) ethanol from molasses. The ethanol yield obtained with the fusant was found to be intermediate between the yields of parental strains. Few years later, the fusion of protoplasts of *S. cerevisiae* and *P. tannophilus* was also performed by Heluane *et al*. [30]. They reported that the hybrid between these two genus can be constructed by protoplast fusion, and the hybrids were found to be able to utilize D-xylose. The field inversion gel electrophoresis of parental and hybrid strains showed that the fusion products had altered genomes. The hybrids morphologically resembled *S. cerevisiae* and exhibited the pattern of sugar assimilation intermediate to parental strains. It was also pointed out by Heluane *et al.* that further work on protoplast fusion of glucose and xylose fermenting yeasts may yield hybrid strains capable of fermenting xylose and some of which may be superior to strains of the known species of xylose-fermenting yeasts. The occurrence of hybrids, constructed by protoplast fusion and their ability to ferment xylose, may be a relatively rare event, but their construction would be worthwhile.

Gupthar et al. performed the fusion of the protoplasts of two different xylose fermenting yeasts *C. shehatae* and *P. stipitis* with ethanol-tolerant *S. cerevisiae* to obtain xylose assimilating hybrids but the fusion resulted in mononucleate fusants which subsequently dissociated into a mixture of parental-type segregants after few sub-culturing [31]. Later, in a study it has been reported that the problem of stability of the fusants can be overcome by carrying out mutations of the fusants [32].

The fusion of protoplast of *S. cerevisiae* with different xylose-fermenting yeasts *C. shehatae*, *P. tannophilus* and *Yamadazyma stipitis* was also reported but the efficiency of fusants in fermenting glucose and xylose was found to be intermediate between both the parents [33]. However, recently successful development of high ethanol yielding fusant from *S. cerevisiae* and *C. shehatae* has been reported by Pasha *et al*. [34]. The fusant strain was reported to utilize xylose as substrate and produce ethanol. The stability of the fusants was further improved by mutagenesis. The developed hybrid strain produced ethanol from the hydrolysates of *Prosopis juliaflora* biomass with a yield of $0.431 gg^{-1}$.

Recently, the development of a genetically stable, high ethanol-producing strain GS3-10 using three rounds of genome shuffling has been reported [35]. The strain was developed by three rounds of protoplast preparation, regeneration, inactivation and fusion using *S. cerevisiae* W5 strain. The high ethanol-producing strain GS3-10, fermented xylose and glucose with 47.08% greater efficacy than that of strain *S. cerevisiae* W5.

Mutagenesis

Mutation is one of the efficient methods to induce change in the genomic composition of any microorganism. In recent years, researchers have reported the improvement in ethanol fermentation and stress tolerance using ultraviolet (UV) radiations [36] and chemical mutagenesis of yeast strains [37]. The various mutagens like UV, ethyl methane sulfonate (EMS) and N-methyl N-nitro N-nitrosoguanidine (MNNG) treatment influence the metabolic activities in yeast with different mechanisms [37, 38]. EMS alkylates the base pairs and brings out their transition from A-T to G-C causing point mutagenesis. MNNG induces transition, transversion, base pair substitutions and large deletion or rearrangements. Further, it reacts with DNA and cause a signature type of damage, producing a variety of lesions which include O^6-methylguanine (O^6-meG), 3-methyladenine and 7-methylguanine. These lesions are usually removed either directly in the first case by a methyltransferase, or through base excision repair using specific glycosylases. UV irradiation induces mitotic crossing over, mitotic gene conversion and reverse mutation by formation of cyclobutane dimers in *Saccharomyces cerevisiae* [39].

Mobini-Dehkordi et al. reported enhancement of 17.3% in ethanol production by mutant *S. cerevisiae* developed using EMS as mutagenic agent [37] while Pasha *et al*. performed mutation of protoplast fusants using MNNG and UV as mutagenic agents and the developed hybrids showed an ethanol yield of 0.49 gg^{-1} using *P. juliaflora* biomass as feedstock. Later the use of UV radiations to develop mutants that are thermotolerant, osmotolerant and ethanol tolerant was reported by Sridhar et al. They found that higher amount of ethanol was produced by mutants than the individual parents at 42 ^{o}C using high glucose concentrations. Their results indicated that UV mutagenesis can be used for improving yeast strains [34, 36].

Recently, the concept of sequential use of multiple mutagens is highly focused as the multiple mutagenesis may alter the multiple genes and thus different physiological characteristics of yeast strains. The concept of multiple mutagenesis has been successfully applied on *Candida tropicalis* for xylitol production using UV and N-methyl-N'-nitro-N-nitrosoguanidine as mutagens [40]. In another study, an improvement of fungal strain for cellulase production has been achieved using repeated mutagenesis for cellulase production by sequential treatments by two repeated rounds of γ-irradiation, ultraviolet treatment and four repeated rounds of treatment with N-methyl-N'-nitro-N-nitrosoguanidine [41]. A similar work was reported by Pang et al. They performed multiple induced mutagenesis of *Kluyveromyces marxianus* yeast for the improvement of ethanol production. A considerable improvement in ethanol yield was achieved after two cycles of alternate mutagenic treatment with UV irradiation and MNNG [11].

2.5 Biomass conversion process

The process for converting the lignocellulose to bioethanol involves the following steps: (1) pretreatment, removal of lignin to make cellulose and hemicellulose more accessible to hydrolytic enzymes; (2) hydrolysis, depolymerisation of the carbohydrate polymers to produce free sugars; and (3) fermentation of mixed hexose and pentose sugars to produce ethanol.

Pretreatment

Pretreatment refers to the disruption of the naturally resistant carbohydrate-lignin shield that limits the accessibility of enzymes to cellulose and hemicellulose. The pretreatment of any lignocellulosic biomass is an essential step before enzymatic saccharification [42]. Pretreatment affects the structure of biomass by solubilizing hemicellulose, reducing crystallinity and increase the available surface area and pore volume of the substrate. The pre-treatment stage promotes the physical disruption of the lignocellulosic matrix in order to facilitate acid- or enzyme-catalyzed hydrolysis. Pretreatments can also have significant implications on the configuration and efficiency of the rest of the process and, ultimately, the economics of bioethanol production. To assess the cost and performance of pre-treatment technologies, techno-economic analyses have been performed recently [43]. A number of pretreatment methods have been explored in recent years such as alkali or acid pre-treatment [42, 44], steam explosion [45], ammonia fibre explosion [46], supercritical CO_2 treatment [47], ozone pre-treatment [48], and biological pre-treatment [49].

The release of the hemicelluloses from the biomass before hydrolysis of cellulose is an essential step in conversion of lignocellulosics to bioethanol. It has been reported that the rate of xylose decomposition is about three times greater than the rate of glucose decomposition [50]. Therefore, the release of hemicellulose increases the susceptibility of the hydrolysing enzymes to act on the remaining cellulosic residues. The increased enzyme digestibility is directly proportional to the hemicelluloses removal because the removal of hemicelluloses creates large pores in the microfibrils which results in increased cellulose accessibility [51]. The hemicellulose recovered during the pretreatment can be fermented separately by pentose fermenting yeasts. Thus, hemicellulose prehydrolysis has been considered as a recovery as well as a pretreatment process [52].

Kadam et al. reported that the pretreatment is the first step required to fractionate lignocellulosic materials into its major plant components of lignin, cellulose and hemicellulose [53]. But, the mechanisms by which pretreatments improve the digestibility of lignocelluloses are however not well understood. However, an important goal of pretreatment is to increase the surface area of lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Besides, increase in surface area, improvement in pretreatment

effectiveness and hydrolysis has been correlated with the removal of hemicelluloses and lignin and the reduction of cellulose crystallinity [53].

A study on the recent developments in key technologies in cellulosic ethanol production has been reported by Lee *et al*. [52]. They discussed the various pretreatment techniques based on the composition of lignocelluloses biomass and simultaneous saccharification and co-fermentation for cellulosic ethanol production. Among all pretreatment methods, dilute acid pretreatment has been widely studied because it is an effective and inexpensive [54]. The dilute sulfuric acid pretreatment can effectively solubilise hemicellulose into monomeric sugars (arabinose, galactose, glucose, mannose, and xylose) and soluble oligomers, thus improving cellulose conversion. Compared to other pretreatment methods, it is especially useful for the conversion of xylan in hemicellulose to xylose that can be further fermented to ethanol by a variety of microorganisms [51]. The removal of 70-92% of xylan was reported by dilute acid pretreatment of various biomass like wheat straw and aspen wood [55], hardwoods and herbaceous crops [56], rye straw and bermuda grass [54], silvergrass [57], wheat straw [42].

Detoxification

The depolymerization of hemicellulose during acid pretreatment process yields xylose as the major fraction and arabinose, mannose, galactose, and glucose in smaller fractions in addition to various microbial inhibitors [58]. These inhibitors can be divided into three major groups, i.e. organic acids (acetic, formic and levulinic acids), furan derivatives [furfural and 5-hydroxymethylfurfural (5-HMF)], and phenolic compounds. The inhibitors affect the physiology of yeast cell which results in decreased viability, ethanol yield, and productivity [58, 60].

Various methods have been investigated for the removal of fermentation inhibitory compounds like overliming [58], ethyl acetate extraction [59], activated charcoal adsorption [61], and laccase oxidation treatment [58]. The effectiveness of the detoxification method depends on the hemicellulosic hydrolysate and the microorganism employed for fermentation [58]. Among the various detoxification methods, overliming and activated charcoal adsorption methods are widely used either individually or in combination [62, 63]. Over-

liming at high pH and temperature has been considered as a promising detoxification method for dilute sulfuric acid-pretreated hydrolysate of lignocellulosic biomass. The detoxification of hemicellulose hydrolysates, by activated charcoal is reported as a cost effective with high capacity to absorb compounds without affecting levels of sugar in hydrolysate [58, 61].

Delignification

An appropriate delignification strategy is essential to facilitate the enzyme hydrolysis of cellulosic biomass as lignin hinders the saccharification process. The cellulase components such as β-glucosidase and endoglucanase shows higher binding affinity towards lignin compared to carbohydrates, which in turn lowers the saccharification efficiency [64]. Therefore, removal of lignin is very important step in the bioconversion of biomass to ethanol. Various delignification approaches have been exploited in the past such as alkali pretreatment [44], hydrogen peroxide pretreatment [65], sulphite pretreatment [67], ammonia fiber expansion pretreatment [46] and sodium chlorite pretreatment [63].

The use of sodium sulphite alone or in combination with sodium chlorite as delignifying agent simultaneously results in swelling of the biomass and enhances the surface area of the substrate accessible to enzymes [67]. According to Gupta et al., sodium chlorite is an elemental chlorine free compound which produces chlorous or hypochlorous acids on heating. The hypochlorous acid then oxygenated chlorous acid resulting in formation of chlorine dioxide that acts as a delignifying agent and depolymerises the lignin [63]. The use of hydrogen peroxide as delignifying agent was explained by Sun et al. [65]. According to them, the delignifying action of hydrogen peroxide is attributed to the hydrogen anion (HOO^-) which eliminates chromophoric groups from lignin.

Enzymatic hydrolysis

The enzymatic hydrolysis of the cellulose component to liberate glucose for ethanol fermentation is one of the major barriers for the process to be economically competitive because of the recalcitrance of feedstock [51]. The cellulose-hydrolysing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases. The endoglucanases catalyse random cleavage of

internal bonds of the cellulose chain,while cellobiohydrolases attack the chain ends, releasing cellobiose. β-glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose. The complete hydrolysis of cellulose and hemicellulose requires a well-designed cocktail of enzymes consisting of endoglucanases, cellobiohydrolases, β-glucosidases, xylanases, mannanases and various enzymes acting on side chains of xylans and mannans [64].

A variety of microorganisms including bacteria and fungi were reported to be able to degrade cellulosic biomass to glucose monomers. Cellulolytic enzyme systems from the filamentous fungi, especially *Trichoderma reesei*, contain two exoglucanases or cellobiohydrolases (CBH1 and CBH2), at least four endoglucanases (EG1, EG2, EG3, EG5), and one β-glucosidase. These enzymes act synergistically to catalyse the hydrolysis of cellulose. The enzyme systems of *Trichoderma reesei* have been most extensively investigated. This species produces numerous cellulose- and hemicellulose-degrading enzymes. However, it is important to supplement *T. reesei* cellulases with extra β-glucosidase activity in order to obtain high cellulose conversion. Recently recombinant cellulases are used to enhance enzymatic hydrolysis of biomass [66].

Few obstacles that prevent the completely functionality of the cellulases are the irreversible adsorption of cellulase to non-polysaccharide components of biomass and the inihibition of hydrolysis reaction by intermediate products like cellobiose. Keeping this in view few innovative strategies to enhance the efficiency of enzymatic hydrolysis has been reported recently which include multistage hydrolysis, new enzymatic mixtures and the use of surfactants [67]. Multistage hydrolysis prevents reaction inhibition due to product accumulation while the use of surfactants checks the unnecessary adsorption of enzymes on lignin.

Various chemical and biological surfactants have been reported to facilitate the conversion of cellulose and thereby enhancing the sugar yield by enzymatic hydrolysis. The use of Tween 20 has been reported to increase cellulose hydrolysis of lime pretreated corn stover by 32% [68], while a combination of Tween 20 and Tween 80 enhances cellulose conversion by 40% [69]. The use of PEG has been reported to alter the ultrastructure of the biomass substrate and

improve enzymatic digestibility [70]. The addition of PEG was found to increase enzymatic conversion of cellulose to glucose from 40% to 80% [70].

Fermentation of lignocellulosic substrates

Acidic and enzymatic hydrolysis of lignocellulosic biomass results in the generation of sugar monomers, principally D-glucose and D-xylose. During fermentation of lignocellulosic substrates both pentose and hexose sugars are converted to ethanol under anaerobic or aerobic conditions. To achieve successful fermentation of these hydrolysates the requirement of an effective yeast strain is of paramount importance. The commonly used glucose fermenting yeast *S. cerevisiae* is not able to ferment xylose while the xylose fermenting yeasts are highly susceptible to inhibitory by-products generated during biomass pretreatment sugar degradation by-products. Therefore, a few attempts have been made by the researchers to develop yeast strains capable of fermenting both hexose and pentose sugars present in lignocellulosic hydrolysates as summarized in Table 1.

Table 1: The fermentation of hydrolysate from various yeasts strains reported

Yeast	**Hydrolysate**	**Fermentation mode**	**Ethanol yield (gg^{-1})**	**Reference**
S.cerevisiae TMB3400	Spruce	Fed batch	0.43	[60]
S.cerevisiae TMB3400	Corn stover	Fed batch	0.33	[60]
S.cerevisiae TMB3006	Spruce	Fed batch	0.37	[60]
P.stipitis CBS 5773	Spent sulphite liquor	Continuous	0.35	[60]
S.cerevisiae 424A(LNH-ST)	Corn stover	Batch	0.33	[71]
S.cerevisiae 424A(LNH-ST)	Corn fiber	Batch	0.41	[71]
MT8-1/Xyl/BGL	Wood chip hydrolysate	Batch	0.41	[72]
F12	Still bottoms fermentation residue	Batch	0.27	[73]
S. cerevisiae 1400(pLNH32)	Corn fiber	Batch	0.49	[74]
P.stipitis NRRL Y-7124	Sunflower seed hull	batch	0.41	[75]

The fermentation of xylose and glucoses rich hydrolysate obtained from acid pretreatment and enzymatic hydrolysis can be performed in separate vessels using xylose and glucose fermenting yeasts respectively. However, the use of hybrid yeasts that are able to co-ferment hexose and pentose sugars facilitates the fermentation of the mixed hydrolysates in a single vessel [59]. The ethanol production from acid and enzymatic hydrolysates of *L. camara* by separate fermentation has been carried out by Kuhad et al. and the ethanol yield obtained from acid and enzymatic hydrolysate were 0.32 and 0.48 gg^{-1}. While Pasha et al. reported an ethanol yield of 0.431gg^{-1} by fermentation of mixed hydrolysates of *L. camara* to ethanol [59].

In an another study, Saha *et al*. conducted dilute pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol and reported an ethanol yield of 0.24 gg^{-1} from acid pretreated enzyme saccharified wheat straw hydrolysate using recombinant *Escherichia coli* strain FBR5 [42]. Bioethanol production from acid pretreated water hyacinth was performed by Satyanagalakshmi *et al*. by separate hydrolysis and fermentation. The various process parameters of enzymatic hydrolysis were optimized and 0.29% (w/v) ethanol was obtained at optimum conditions [76]. In the same year Sindhu et al. performed the dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. The fermentation of the hydrolyzate using *Saccharomyces cerevisae* has been reported to produce 11.365 gL^{-1} of bioethanol with an efficiency of about 50% [77].

Kim *et al*. tried dilute acid pretreatment, saccharification and fermentation of barley straw. They reported that the fermentation of glucose and xylose attained a level 90% of that of the theoretical maximum of ethanol at 12 h using *S. cerevisiae* K35 and *P. stipitis* KCCM 12009 [78]. Sindhu *et al*. utilized sugarcane tops as substrate for bioethanol production and reported an ethanol production of 11.37gL^{-1} from cellulosic hydrolysate using *S. cerevisiae* strain [77].

Chapter 3

SCOPE AND OBJECTIVES

From literature it is evident that there is increasing demand of bioethanol, as it is considered as a promising and environmental benign alternative energy sources for transportation sector. Lignocellulosic biomass is the most potential feedstock for the production of bioethanol because of its widespread availability, sustainable supply and low cost. In this context, one of the key challenges is the development of an efficient microbial strain that can co-ferment pentose and hexose, the major sugar components present in this lignocellulosic biomass exploitation of this strain for actual bioethanol production.

Therefore, the main objective of the present research is the development of efficient hybrid yeast strain and utilization of this strain for the production of bioethanol from lignocellulosic biomass.

The specific **objectives** of the present research work are as follows:

1. To develop hybrid yeast strain comprising of glucose and xylose fermenting yeast strains by protoplast fusion technique.
2. To improve the industrially important characteristics of hybrid strain by sequential mutagenesis.
3. To evaluate the performance of hybrid strains towards bioethanol production using glucose-xylose mixture as model lignocellulosic substrates.
4. To investigate the bioethanol production from lignocellulosic biomass using developed hybrid strains
5. To optimize the key process parameters that will facilitate the ethanol production.

Scope of work

Recently research workers published data on the development of hybrid strains by protoplast fusion of the most industrially important yeast strain *Saccharomyces cerevisiae* and potential xylose fermenting yeasts such as *C. shehatae, P. tannophilus, and P. stipitis* with the intention to produce bioethanol from lignocellulosic biomass. However the development of hybrid yeast strains have been attempted by a few researchers, the study on bioethanol production, particularly from lignocellulosic biomass using these strains in inadequate. Therefore, there is

a need of research program for a systematic study on the development and exploitation of this potential strain for actual production of bioethanol from lignocellulosic substrates.

The present investigation is therefore, aimed to develop efficient hybrid yeast strain and investigate the influence of key parameters on the performance of these strains on the bioethanol production from potential lignocellulosic biomass. The entire investigation has been divided into the following stages:

1. **Development of hybrid yeast strain by protoplast fusion**

   In this part, various hybrid strains comprising of widely used glucose fermenting yeast *S.cerevisiae* and three xylose fermenting yeast strains such as *P. tannophilus, P. stipitis, C. shehatae* were developed by protoplast fusion. The fusants were sorted using FACS as a rapid method and confirmed by molecular characterization such as RAPD and DNA sequencing techniques.

2. **Evaluation of hybrid strains for ethanol production**

   In this area, the efficacies of the various hybrid strains were tested through fermentation experiment using glucose-xylose mixture as model lignocellulosic substrates. Based on the ability to ferment sugar mixture with high efficiency, the most efficient strain was selected for further study.

3. **Improvement in stress tolerance of hybrid strain by sequential mutagenesis**

   During the fermentation process, the yeast undergoes various kinds of stresses that affect the yeast growth and ethanol production. Therefore, in this part of research work, effort has been given for the improvement of various stress tolerance of the developed fusant strain by mutagenesis to facilitate ethanol production.

4. **Stress tolerance and stability study**

   The mutants obtained by sequential mutagenesis were evaluated for their tolerances to different stresses such as ethanol, thermal and inhibitor stress and the yeast showing maximum stress tolerance and stability was selected for the fermentation of the multiple sugars under stress conditions.

5. **Evaluation of mutant strain by ethanol production from glucose-xylose mixture**

   The selected mutant strains was further evaluated for their efficiency in producing ethanol under individual and combined stress factors by conducting the fermentation reaction of glucose-xylose mixture.

6. **Study on the production of bioethanol from *L. camara* and *I. carnea* biomass using hybrid strain**

   This is the broad area of the present investigation. In this phase of the research work, a detailed study on the conversion of potential lignocellulosic biomass e.g. *Ipomoea carnea* and *Lantana camara* to bioethanol was undertaken. Various steps involved in the conversion of biomass to fermentable sugars such as pretreatment, detoxification, delignification and enzymatic hydrolysis were optimized to achieve maximum release of sugars. Finally the fermentation experiments were carried out to convert the released sugar to bioethanol. The parametric sensitivity of the fermentation process was also studied elaborately to maximize the sugar conversion, ethanol yield and productivity. A comparison of the efficiency of the hybrid yeast strain developed in this study with the other hybrid strains reported was also done.

Chapter 4

EXPERIMENTAL DETAILS

4.1 DEVELOPMENT OF PROTOPLAST FUSANTS

4.1.1 Yeast strains

Saccharomyces cerevisiae, the glucose fermenting yeast strain (NCIM-3090) and three xylose fermenting yeast strains namely *Pachysolen tannophilus* (NCIM-3502), *Candida shehatae* (NCIM-3500) and *Pichia stipitis* (NCIM-3507) were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. The yeast cultures were maintained on YPD agar slants at 4^{o}C.

4.1.2 Media and buffers

The media and buffers used in the process of protoplast formation and fusion are listed in Tables 2 and 3. The media and buffers were prepared with utmost accuracy of weights and volumes.

Table 2: Media composition

Sl. no.	Media	Composition	pH
1.	YPD agar	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 15 g L^{-1} agar and 20 g L^{-1} dextrose	pH 6.5
2.	YPD broth	10g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose	pH 6.5
3.	YPX broth	10g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} xylose	pH 6.5
4.	Regeneration media	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 18 g L^{-1} agar and 20 g L^{-1} dextrose,0.67% yeast nitrogen base and 0.6M KCl	pH 6.0
5.	Regeneration overlay media	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 10 g L^{-1} agar and 20 g L^{-1} dextrose,0.67% yeast nitrogen base and 0.6M KCl	pH 6.0
6.	Fermentation media	2 gL^{-1} yeast extract, 0.4 gL^{-1} $MgSO_4$, 2gL^{-1} $(NH_4)_2SO_4$, 5gL^{-1} KH_2PO_4 and 200 gL^{-1} glucose–xylose mixture (3:1 ratio)	pH 4.5

Table 3: Buffers and their composition

Sl. no.	Buffer	Composition	pH
1.	0.1M Potassium phosphate buffer	0.1M potassium hydrogen di-phosphate and 0.1M potassium di-hydrogen phosphate	7.0
2.	Cell wall lysis buffer	3 mg/mL lysing enzyme from *Trichoderma harzianum* and 0.6 M of sorbitol dissolved in 0.1M phosphate buffer	7.0
3.	STC buffer	0.6 M sorbitol; 10 mM Tris-HCl; 10 mM $CaCl_2$	7.5
4.	Protoplast fusion buffer	33% PEG (MW 4000) in STC buffer	7.5

4.1.3 Enzymes and chemicals

The lysing enzyme from *Trichoderma harzianum* and fluorescent dyes (FITC and PI) were purchased from Sigma-Aldrich Co. (St. Louis, USA). The other chemicals were purchased from either Merck (Darmstadt, Germany) or Himedia (Mumbai, India).

4.1.4 Protoplast formation

Yeast cultures were grown in YPD medium for 14 h at 30^{o}C and 120 rpm. The cells were harvested by centrifugation at 3000 rpm for 10 min and the pelletized cells were washed with distilled water followed by 0.1M potassium phosphate buffer. The cells were re-suspended in cell wall lysis buffer and the cell suspension was incubated at 30^{o}C and 75 rpm for 4 h. The lysis of cell wall and release of protoplast were monitored under phase contrast microscope (Axiovert 40C, Carl Zeiss, Germany) at an interval of 30 min and the number of protoplast formed was counted using haemocytometer. The effects of various key parameters of protoplast formation such as lysis duration (1-4 h), lysing enzyme concentration (1-4 mg/mL) and osmotic stabilizers (KCl, sorbitol, and $MgSO_4$) were investigated and optimum conditions of protoplast formation were established. The lysis of cell wall was also observed under scanning electron microscope (JSM-6480 LV, Jeol Co., Japan) for detailed examination of the

effect of lysing enzyme on the yeast cell wall. After the formation of protoplasts, the cell wall debris were removed by centrifuging the suspension at 500 rpm for 15 min and the protoplasts formed were suspended immediately in buffer-osmotic stabilizer solution (0.6 M of sorbitol in 0.1M phosphate buffer).

Protoplast yield was calculated using the formula [79]

$$\textbf{Protoplast yield} = \frac{\textbf{Number of protoplasts released}}{\textbf{Number of cells incubated with lysing enzyme}} \textbf{ X 100}$$

4.1.5 Fluorescence labelling of protoplasts

The released protoplasts of *S. cerevisiae* and xylose fermenting yeasts were labelled with two different fluorescent dyes with the aim of sorting the fusant hybrids based on dual fluorescence. The dyes fluorescein isothiocyanate (FITC) (50 µg/mL in DMSO) and rhodamine 6G (R6G) (100 µg/mL in acetone) were used for fluorescent labelling. 20 µL FITC was added to 1 mL of buffer containing10^6 protoplasts of *S. cerevisiae* and incubated in dark for 30 min. The protoplasts of three xylose fermenting yeasts were similarly stained with 15µL R6G for 1 h. After fluorescent labelling of cells the extra dye was removed by washing the cells twice with buffer-osmotic stabilizer solution.

4.1.6 Protoplast fusion

The fusion of labelled protoplasts was carried out following the method described in published literature [80]. Equal volume of protoplast suspension of *S. cerevisiae* and xylose-fermenting yeast each containing 10^6 numbers of fluorescent labelled protoplasts were mixed and centrifuged at 500 rpm for 5 min. With the aim to optimize most favourable conditions for maximum fusion of protoplasts, the pellet was re-suspended in fusion buffer at varying concentrations of PEG in the range 25-45% (w/v) and pH in the range (5-5.8). The suspension was incubated at 30°C and 100 rpm. 0.25 mL of the suspension was taken out after every 5 min, the fusion of protoplasts was monitored under phase contrast microscope and the number of fused protoplasts were counted using haemocytometer.

The fusion frequency was determined by the formula

Fusion frequency = Number of protoplasts fused/ Total number of protoplasts X 100

4.1.7 Sorting of fusants

The fusants were sorted using a fluorescent activated cell sorter (FACS Aria III, Becton-Dickinson) equipped with an argon-ion laser as the light source. The beam power level and the flow rate were kept at 50 mW and 500 s^{-1}. The parental strains showed green and red fluorescence in different gates based on the staining with fluorescent dyes FITC and R6G. Subpopulation of cells exhibiting fluorescence in both FITC and R6G regions were sorted from the dual positive quadrant.

4.1.8 Regeneration of fusants

The fusants were washed with STC buffer and regenerated on regeneration medium plates. 0.5 mL of suspension of fused protoplasts was mixed with 10 mL of osmotically stabilized melted regeneration overlay medium and poured as a thin top layer on the plate with regeneration medium. The plates were then incubated at 28^{o}C until colonies appeared. The colonies grown on regeneration plate were replica plated on YPD and YPX plates containing glucose and xylose as carbon source respectively to confirm their hybrid nature.

4.1.9 Stability study

The stability of fusants was examined after every 15 days for a period of 9 months by assessing their substrate utilization and ethanol fermentation efficiencies using glucose-xylose mixture. The substrate utilization was assessed by routinely spreading the strain for a single colony on YPD and YPX agar plates. After 24 h growth, the cells were inoculated in fermentation media containing glucose-xylose mixture and the ethanol production was measured. The fusants showing same efficiency of substrate utilization and ethanol production were considered as stable.

4.1.10 Genetic Characterization of fusants

Relative DNA content of fusant

To study the relative DNA content of fusants, one cycle of fusion experiment was conducted without sorting. The DNA content of the cells was estimated using the method reported

Mukai et al. [81]. The fusant and parental cells were fixed in 95% (v/v) ethanol overnight. The cells were washed with 50 mM Tris-HCl buffer (pH 8.0) and treated with Tris-HCl buffer containing 1 mg/mL RNase for 1h at 37^{o}C. The cells were then washed with buffer and stained with 50 µg/mL propidium iodide (PI). The relative DNA content of the cells was analyzed using flow cytometry (FACS LSR Fortessa, BD Biosciences) at excitation wavelength 488 nm and emission wavelength of 610 nm.

Random amplified polymorphic DNA

The fusants were characterized by RAPD (random amplified polymorphic DNA) and DNA sequencing techniques. RAPD was done to evaluate the similarity between fusant, mutant and parents strains. Genomic DNA was isolated from yeast samples using Chromous genomic DNA isolation kit (RKT09, Bangalore, India) according to the standard protocol. The PCR amplification of the isolate was performed using RAPD primer 5'-CAC AGA ATA TMA TCR CYC WC-3'. The amplified DNA fragments were separated by electrophoresis on 1.5% agarose gel with ethidium bromide. The DNA fragments were scanned under UV, photographed and analysed. A 100bp and 500 bp DNA ladders were used as the size standard.

DNA sequencing

The ITS 1, 5.8S ribosomal RNA gene and ITS 2 region of fusant, mutant and parental strains were partially sequenced using the genomic DNA of these strains as a template. PCR product generated using the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was sequenced using Genetic analyzer (Applied Biosystems) and multiple sequence alignment was done using CLUSTAL X.

4. 1.11 Evaluation of fusants by fermentation of glucose-xylose mixture

The fusants and parental yeast strains were grown in 250 ml Erlenmeyer flask containing 200 mL YPD broth (pH 6.5) at 30°C and 120 rpm for 72 h. 10 mL of inoculum was added to the fermentation medium containing glucose–xylose mixture (3:1 ratio) as sugar substrate. The composition of the fermentation medium is mentioned in table 2. The fermentation broth was withdrawn after every 4h and analyzed for ethanol, cell mass and residual sugar content.

4.2 IMPROVEMENT OF HYBRID STRAINS BY MUTAGENESIS

4.2.1Chemicals and media

The chemical mutagens namely EMS and MNNG and fluorescent dye Flourescein di-acetate (FDA) were purchased from Sigma-Aldrich. Rest of the chemicals were purchased from Merck. The fermentation media and buffers used in the study are presented in Table 2 and 3.

4.2.2 Sequential mutagenesis

Mutagenesis of the fusant strain was conducted by eight different combinations of mutagenic treatments using EMS, MNNG, near UV and far UV radiations as mutagens. The fusant yeast cells grown overnight in YPD medium were harvested and washed with 0.1M sodium phosphate buffer (pH 6.8). Cells were sonicated for 5 sec and re-suspended in the buffer. Chemical mutagenesis was carried out by adding mutagenic agent (50 µL EMS or 50 µg MNNG) to 2 mL of cell suspension. The suspension was then incubated at 30^{o}C with shaking at 120 rpm. After 45 min, 0.5 mL cell suspension was taken out, washed with 3.0 mL of sodium thiosulfate (5% w/v) and resuspended in buffer.

Mutagenesis of fusant was also induced by far (254 nm) and near (290-350 nm) ultraviolet radiations using UV lamps (30W, Philips, India). About 2 mL of cell suspension in buffer was transferred to sterile petriplate and exposed to UV radiation for 90 sec. After serial dilution in buffer, cells were plated on YPD plates and incubated at 30^{o}C. Colonies with better morphology and size were selected from the plate and analysed for stress tolerance studies and ethanol production.

4.2.3 Cell Viability assay

After each stage of mutagenesis, the viability of cells was checked by FDA-PI dual staining to assay the lethality of mutagen. The cells were washed and suspended in phosphate buffer. 50 µL of FDA solution (5 mg/mL in acetone) was added to 1mL of cells suspension, the resulting suspension was centrifuged at 500 rpm for 3 min and the cells were incubated in dark for 5 min. The cells were then stained with propidium iodide (PI) by adding 20 µL of PI per mL of cell suspension. The viability of cells was assayed using flow cytometer (LSR

Fortessa, BD Biosciences) and the LD 50 (Lethal dose 50) was estimated after each step of mutagenesis.

4.2.4 Stress tolerance study

The stress tolerance in mutants was evaluated by conducting fermentation experiment using glucose-xylose mixture as model lignocellulosic substrates (3:1 ratio). The fermentation temperature and agitation were kept at 30^{o}C and 150 rpm throughout the fermentation experiment. The ethanol tolerance and thermotolerance were examined by growing mutants in YPD medium containing different concentrations of ethanol (6-10% v/v) and temperature range (38-42^{o}C) respectively. The tolerance of the mutants to toxic inhibitors was studied by adding known amount of inhibitors (vanillin, furfural and acetic acid) in the fermentation medium. Mutant grown at 30^{o}C in YPD medium containing 175 gL^{-1} glucose-xylose mixture without ethanol or inhibitors was used as control. The study of combined effect of thermal and inhibitor stress was carried out by fermentation of 150 gL^{-1} glucose–xylose mixture at 39^{o}C and 150 rpm in presence of inhibitors. The combined effect of ethanol and inhibitor stress was investigated by carrying out fermentation experiment at 30^{o}C using fermentation medium supplemented with 5% ethanol and the three fermentation inhibitors. Furthermore, the combined effect of thermal, inhibitors, ethanol and substrate stress was investigated with the fermentation medium containing 250 gL^{-1} glucose–xylose mixture, 5% ethanol and fermentation inhibitors at 39^{o}C. The amount of inhibitors used were 0.25-1.0 gL^{-1} vanillin, 0.25-2.0 gL^{-1} furfural and 2-8 gL^{-1} acetic acid in each fermentation experiment. The range of the inhibitors was selected on the basis of published literature [82-84]. The range of temperature and ethanol concentrations has also been selected based on the published literature and the knowledge of the most optimum conditions for yeast growth [36, 85].

4.2.5 Evaluation of ethanol production of mutants under normal conditions

The performance of the mutants towards ethanol production was studied by fermentation of glucose-xylose mixture in a 250 mL Erlenmeyer flask at 30^{o}C and 150 rpm. The fermentation medium with 175 gL^{-1} glucose-xylose mixture (3:1 ratio) was used for fermentation unless

otherwise specified. Initial pH and inoculum size were maintained at 4.5 and 10% respectively.

4.2.6 Trehalose assay

Trehalose content of yeast cells was estimated following the methods previously described by Swan and Watson [86]. In brief, mutant and fusant yeast cells were centrifuged and washed with distilled water and with 0.1M sodium phosphate buffer (pH 6.8). The cells were then suspended in 2 mL buffer in test tubes and the tubes were placed on ice and 4 ml of 0.5M cold tri-chloro acetic acid (TCA) was added to each of them. The cell suspension was shaken gently at intervals of 10 min for 30 min, suspension was centrifuged at 4000 rpm and the supernatant were collected. This step of suspending cells in TCA and collection of supernatant was repeated till about 20 mL of supernatant was obtained. The volume of supernatant was made to 50 mL by addition of distilled water. 1mL of diluted supernatant was added to 5 mL of anthrone reagent and tubes were placed in boiling water bath for 10 min. The optical density at 620 nm was determined for each sample. The trehalose content of each sample was compared with the standard curve and recorded.

4.2.7 Ergosterol content

The ergosterol content of the cells was determined by the spectrophotometric method of Breivik and Owades [87]. The cells grown in YPD media for 18 h were digested in 25% w/v alcoholic KOH at 90^{o}C for 3h. The saponified samples were then extracted into n-heptane and ergosterol content was determined spectrophotometrically at 281.5 nm.

4.2.8 Stability study

The stability of mutants was examined after every 15 days for a period of 9 months by assessing their substrate utilization and ethanol fermentation efficiencies using glucose-xylose mixture as explained in section 4.1.9.

4.2.9 Genetic characterization of mutant

RAPD and partial DNA sequencing of the mutants was done to investigate the changes brought in their genetic constitution after mutagenesis. The detailed procedure of steps of genetic characterization has been mentioned in section 4.1.10.

4.3 BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

4.3.1 Biomass collection and its composition analysis

Ipomoea carnea and *Lantana camara* biomass were collected from the campus of the National Institute of Technology, Rourkela, Odisha, India. The plants stems were cut near to root and the whole plant biomass including leaves, flowers and fruits were used for the experiment. The plant biomass were washed with tap water and dried in shade for 48 h. The dried biomass was cut in to pieces of 2-5cm size and further pulverized in a laboratory grinder (Bajaj Electricals, India) to the size range of 0.5 to 0.75 mm. The powdered biomass was again dried under shade for 6 h at room temperature to remove left over moisture. The biomass was extracted with alcohol–benzene (1:2, v/v) mixture for 4h using Soxhlet extraction apparatus and extractives content was estimated. The chemical composition of the extractive free biomass was assayed.

Moisture content

The moisture content of biomass feedstock was determined by drying the weighed amount of sample in a silica crucible at 105 ^{o}C in a hot air oven till a consistency in weight was observed. The moisture content was determined as follows:

$$\% \text{ Moisture (wet basis)} = ([W_2 - W_f]) / ([W_2 - W_1]) \times 100$$

$$\% \text{ Moisture (dry basis)} = ([W_2 - W_f]) / ([W_f - W_1]) \times 100$$

where W_1 is the weight of empty crucible, W_2 is the weight of the crucible and sample, and W_f is the constant weight of crucible and sample after drying.

Ash content

A known quantity of oven-dried sample was taken in a pre-weighed silica crucible and combusted in a muffle furnace at 750 ± 25 ºC for about 4 h.

The ash content was estimated following the formula:

$$\% \text{ Ash (dry basis)} = ([W_f - W_1]) / ([W_2 - W_1]) \times 100$$

where W_1 is the weight of the silica crucible, W_2 is the weight of the crucible and the oven-dried sample, and W_f is the constant weight of the crucible and the sample after combustion.

Elementary analysis

The determination of carbon (C), hydrogen (H) and nitrogen (N) in biomass is called elementary analysis. These elements were determined by CHNSO analyzer (PerkinElmer 2400) following standard protocol (ASTM 5373). About 1.0 mg of sample was used in a tin boat assortment at 900^{o}C in an oxygen atmosphere, so that the carbon is converted to carbon dioxide, hydrogen to H_2O and N to N_2. The percentage of oxygen was determined by means of difference.

Cellulose

For cellulose estimation, 10 mL acetic acid/nitric acid reagent (15 ml of 80% acetic acid mixed with 1.5 mL of conc. nitric acid) was added to 0.1 g of biomass and the mixture was kept in a water bath for 30 min. After cooling the sample was centrifuged at 8000 rpm for 10 min and supernatant was discarded. The pellet was washed twice with distilled water and 10 mL sulphuric acid (67% v/v) was added and resulting suspension was allowed to stand for 1 h. The suspension was diluted to 5 times (50 mL volume), and 4 mL of anthrone reagent was added to 1 mL of diluted mixture. The mixture was incubated in a boiling water bath for 15 min for colour development. After cooling the tubes, the optical density was read at 620 nm against the reagent blank (1 mL distilled water and 4 mL anthrone reagent). The concentration of cellulose in the sample was calculated using glucose standard.

Hemicellulose

10 mL of 3% (w/v) sulphuric acid was added to a 1 g of oven-dried sample. The samples were autoclaved at 121ºC for 1h and pH was adjusted to 7.0 with potassium hydroxide and hydrochloric acid. The sample was diluted 10 times with distilled water and 1 mL p-bromoaniline reagent was added to a 5 mL of sample. The suspension was kept in a water bath at 70ºC for 10 min and followed by incubating in dark at room temperature for 70 min. The optical density was taken at 520 nm against reagent blank. The concentration of cellulose in the sample was calculated using xylose standard.

Lignin

For lignin estimation, 3 mL of 72% sulphuric acid was added to a 300 mg of biomass sample taken in a tube and the tube was kept in water bath at 30^{o}C for 1 h. The sample was stirred after every 10 min for 1 h for uniform hydrolysis. The sample was diluted to a concentration of 4% by adding 84 mL of water. The mass was then autoclaved for 1h at 121ºC. After cooling, the sample was vacuum filtered. The residue and filtrate were both used for AIL (acid-insoluble lignin) and ASL (acid-soluble lignin), respectively. The residue was oven-dried at 105ºC till a constant weight is achieved and further heated at 550ºC in muffle furnace to determine ash content. AIL was estimated using the following formula

$$\mathbf{AIL}(\%) = \left(\frac{\boldsymbol{Wr}}{\boldsymbol{Ws}}\ \mathbf{x\ 100}\right) - \left(\frac{\boldsymbol{Wa}}{\boldsymbol{Wr}}\ \mathbf{x\ 100}\right) - \%\ \boldsymbol{P}$$

where Ws and Wr are the weights of the untreated sample and oven-dried acid-insoluble residue, Wa is the weight of the ash left after combustion, and P is the protein content in biomass.

The optical density of the filtrate was measured at 320 nm wavelength. The sample was diluted to bring the absorbance to 0.7–1.0, recording the dilution. Four percent sulphuric acid was used to dilute the sample and as blank.

The concentration of ASL was calculated using the following formula.

$$\boldsymbol{ASL}\ (\%) = \frac{\textbf{UV abs X Volume of filterate X dilution}}{\boldsymbol{\varepsilon}\ \textbf{X}\ \boldsymbol{Ws}} \text{x}\ \mathbf{100}$$

where UV_{abs} is the absorbance of sample at 320 nm, volume of filtrate is 87 mL, 'dilution' is the volume of sample of diluting solvent/volume of sample, ε is the absorptivity of biomass at specific wavelength and Ws is the oven-dried weight of sample. Total lignin content was calculated by the sum of AIL and ASL.

4.3.2 Biomass pretreatment

Acid pretreatment

The biomasses were subjected to various dilute acids treatment using sulphuric acid, hydrochloric acid and phosphoric acid. The purpose of this treatment is to select an effective acid which facilitates the maximum release of hemicellulose from biomass. The biomass was soaked in 1-4% (v/v) acid with 10% (w/v) solid loading and autoclaved at 120^{o}C for 1h. The slurry was separated by filtration through double layered muslin cloth and the biomass collected as residue was washed thoroughly with tap water till its pH reached 7.0. The neutralized biomass was dried at room temperature for 2 h and then stored at 4^{o}C in sealed packs until use. The acid hydrolysates and the pretreated biomass obtained after different acid treatment were analysed for the amount of xylose, glucose and pretreatment by-products like furans and phenolics.

Selection of suitable acid for pretreatment was followed by optimization of major pretreatment parameters for biomass pretreatment such as temperature (100-140^{o}C) and time of treatment (30-60 min) to establish the optimum conditions for the release of maximum amount of sugars and minimum pretreatment by-products. The variation in the content of pretreated biomass and acid hydrolysate was studied with respect to varying pretreatment severity.

The pretreatment severity was determined by the combined severity factor (CSF) or severity index according to the formula reported by Lloyd and Wyman, 2005 [88]. The CSF was calculated as

$$\mathbf{CSF = \log \{t. \exp [(T_H - T_R) / 14.75]\} - pH}$$

where t is reaction time in minutes, T_H is the reaction temperature (^oC), T_R is the reference temperature (100^oC) and pH is the acidity of the dilute acid.

Detoxification of acid hydrolysate

The acid hydrolysate was detoxified following to the method described by Buhner *et al*. [89]. The process involved the use of overliming and activated charcoal treatment both individually and in combination. Overliming was performed by the addition of calcium hydroxide to the acid hydrolysate till pH reaches 10.0. The whole mixture was stirred for 30 min at 45^oC and allowed to cool to room temperature. The acid hydrolysate was then neutralized with concentrated H_2SO_4 and centrifuged at 10,000g for 10 min. The supernatant was then mixed with 2.0% (w/v) activated charcoal and the slurry was stirred continuous for 90 min at room temperature. Finally, the detoxified sugar syrup was recovered by vaccum filtration.

Delignification of pretreated biomass

Delignification of the pretreated biomass was carried out to remove the residual lignin from pretreated biomass. The acid pretreated residue was treated with 5-20% (w/v) sodium sulphite at pH 9 with solid loading of 10% (w/v). The mixture was autoclaved at different temperatures in the range $100\text{-}140^oC$ and different time periods (30 and 45 min). The mixture was then filtered and the residue was washed till neutral pH is obtained and stored at 4^oC until further use. The lignin content in the delignified biomass was estimated and the percentage lignin removal was calculated.

Characterization of native, acid pretreated and delignified biomass

The change in surface structures, functional group and crystallinity of native, acid pretreated and delignified biomasses of *I. carnea* were analyzed by SEM, FTIR, and XRD analysis.

SEM study

The structural changes in the surface of pretreated and delignified biomass were studied by analysing images obtained by a scanning electron microscope (JSM-6480 LV, Jeol Co., Japan). The powdered biomass was mounted on a conductive tape and coated with gold palladium. The images were captured at 10-15KV and at magnification ranging from 250x to 1000x depending on the feature to be traced.

FTIR study

The FTIR spectra of biomass samples were obtained by FTIR spectroscopy (Shimadzu IR Prestige 21, France). 10 mg of biomass was mixed with 300 mg of KBr and the mixture was compressed to pellets. The spectra were recorded in the range 400-4000 cm^{-1} with a resolution of 2 cm^{-1}.

XRD study

The crystallinity of native, pretreated and delignified biomass was analyzed by XRD analysis using X ray-diffractometer (Philips PANalytical X'pert pro, Germany) with Cu-Kα radiation at 40 kV, 40 mA, scanning angle of 10-40^o and scanning speed of 0.5^o min^{-1}. The crystallinity of cellulose was calculated according to the empirical method described by Segal et al. for native cellulose [90]. The intensities of the amorphous (2θ = 18^o) and crystal regions (2θ = 22.6^o) were utilized to calculate the Crystallinity index (CrI) as follows:

$$\boldsymbol{Crystallinity\ Index\ (CrI) = \frac{(I_{22.6^o} - I_{18^o})}{I_{22.6^o}}\ x\ 100}\ (\%)$$

where I_{θ}^{o} is intensity at the corresponding θ.

4.3.3 Enzymatic hydrolysis

The enzymatic hydrolysis experiment of the delignified cellulosic residue was carried out in 500 mL Erlenmeyer flasks. The delignified biomass was added to 0.05M citrate phosphate buffer (pH 5.0) on the wet basis with 6.5% solid content. The resulting slurry was autoclaved at 121^{o}C for 15 min. After cooling, cellulase (1.5-3.00 FPU/mL) and cellobiase (3 times of cellulase concentration) were added to the suspension and incubated in a controlled environment incubator shaker (CIS-24 BL, Remi Instruments Ltd., India) at 50^{o}C and 150 rpm for varying time intervals (8-40 h). The effect of surfactants on enzymatic saccharification was studied by adding different surfactants like Tween 20 (T20), polyethylene glycol and Tween 80 (T80). A study on supplementation of BSA with surfactants was also conducted with the addition of 0.25% (w/v) BSA (Sigma–Aldrich, St. Louis, MO, USA) 1h prior to the addition of enzymes and surfactants. Samples were withdrawn after every 4 h interval and analysed for reducing sugar released in the reaction mixture.

The saccharification efficiency was calculated using the formula reported by Kuhad et al. [67].

Saccharification efficiency (%) = {Amount of glucose released /total sugar concentration in the pretreated substrate} X 100

The optimization of the key parameters of the enzymatic hydrolysis experiment was done using response surface methodology. A Box–Behnken design [91] was used to determine the effects of independent parameters and their interactions on the response. The four parameters were biomass loading, enzyme loading, surfactant concentration and temperature. The results were analyzed by MINITAB 16 software (Minitab Inc., USA).

4.3.4 Ethanol fermentation

A 5 litre bench top fermenter, model Biostat B plus (Sartorius, Bangalore, India) equipped with standard control and instrumentation was used for the large scale fermentation of ethanol. The fermenter was equipped with an agitator consisting of flat bladed impeller with

three blades, digital controller, aeration tube with sparger and sensors for temperature, pH, dissolved oxygen, foam and level. Three 500 ml bottles and silicone tubes, provided for addition of acid, base and antifoam agent to the fermenter are operated by peristaltic pump. The temperature was maintained by powerful heater and automatic controlled cooling water valve. The fermenter vessel containing the fermentation media was autoclaved for sterilization at 15 psi for 30 min. The sterilized vessel was connected to the main assembly using water and air tubes. The probes for pH, temperature and dissolved oxygen were standardized and the inoculum was added using syringe. During fermentation the pH was maintained using sterile 1M NaOH and HCl. The bioreactor setup is shown in fig 5.

Salient features of bioreactor

a) 5 L scalable glass culture vessel
b) Digital controller of Agitation Speed, pH, DO, Temperature, Foam, Level, Substrate Addition, Gas Mixing and Gas Flow Rate
c) Agitation motor
d) Agitator
e) Temperature probe
f) pH probe
g) DO probe
h) Acid and base
i) Peristaltic pumps
j) Gas flow system
k) Sampling system

Fig. 5: Bioreactor setup for ethanol fermentation

2L each of detoxified acid hydrolysate and enzymatic hydrolysate were mixed along with the supplementation of 3.0 gL^{-1} yeast extract, 2.0 gL^{-1} KH_2PO_4, 1.0 gL^{-1}peptone, 0.5 $gL^{-1}MgSO_4.7H_2O$, 0.5 gL^{-1} NH_4Cl, 0.25 gL^{-1} $(NH_4)_2HPO_4$, 0.1 gL^{-1} $CaCl_2.2H_2O$ and 0.1 gL^{-1} $FeCl_3.2H_2O$. The fermentation of mixed hydrolysate was conducted using 10% inoculum of mutant yeast strain RPRT90 at 30^oC, 5.5 pH, 150 rpm and 0.3 litres per min aeration for 36 h. The fermentation broth was then centrifuged. The supernatant was used for estimation of ethanol and residual sugar and the biomass was estimated from the pellet.

4.3.5 Analytical methods

Ethanol estimation

Ethanol was enzymatically estimated as per the method described by Puria *et al*. [92] In this assay, fermentation broth was centrifuged and the supernatant was diluted 10 times with 50 mM sodium pyrophosphate buffer (pH 8.8). 0.65 ml sodium pyrophosphate buffer (50 mM), 0.75 ml β–NAD (15 mM) and 50 µL alcohol dehydrogenase enzyme (3.0 U) were added to 50µL diluted sample and optical density at 340 nm was measured using spectrophotometer. BSA was used in the place of enzyme in blank. The ethanol concentration in the sample was calculated using a standard graph.

Sugar estimation

The sugar was estimated by DNS method [93] and the amount of residual glucose was separately estimated using glucose-oxidase based assay kit (Sigma Chemical Co.). The estimation of sugars in hydrolysate and biomass was done following NREL protocols using HPLC (Shimadzu Corp., Kyoto, Japan) with an Aminex HPX-87H organic analysis column (300 x7.8 mm, Bio-Rad Hercules, CA) and a refractive index detector. 4mM H_2SO_4 was used as eluent and flow rate was maintained at 0.65 mL/min at 65^oC.

Biomass estimation

The cell mass concentration was estimated by dry cell mass weight measurement. The pellet obtained by centrifugation of fermentation broth was dried at 70^{o}C till constant weight is achieved. The biomass was also estimated by measuring optical density at 620nm.

Estimation of inhibitor

Total phenolics released during acid pretreatment of lignocellulosic biomass were determined by Folin–Ciocalteu reagent method [94] using vanillin as standard. Total furans in hydrolysate were assessed by a spectrophotometric method based on the difference in absorbance at 284 and 320 nm [95] before and after the pretreatment.

Chapter 5

RESULTS AND DISCUSSION

Part I

DEVELOPMENT OF HYBRID YEAST STRAINS BY PROTOPLAST FUSION

5.1 DEVELOPMENT OF HYBRID YEAST STRAINS BY PROTOPLAST FUSION

5.1.1 Introduction

It is evident from literature review that the lack of efficient microorganisms to ferment a variety of sugars released by the hydrolysis of lignocellulosic materials is one of the major factors limiting the complete utilization of lignocellulose for bioethanol production [96]. Moreover, the most commonly used glucose-fermenting yeast strain; *S. cerevisiae* is not able to ferment pentose sugars present in lignocellulosic biomass [5]. Therefore, the recent research has been focused on the development of hybrid strains by protoplast fusion of *S. cerevisiae* and various potential xylose-fermenting yeasts.

In this phase of work, effort has been given on the development of hybrid yeast strains from the widely used glucose fermenting yeast *S. cerevisiae* and three potential xylose fermenting yeasts, *Pachysolen tannophilus, Candida shehatae and Pichia stipitis* by protoplast fusion technology. The potentiality of the developed hybrid strains in producing ethanol was evaluated by fermentation experiment using a mixture of glucose and xylose as model lignocellulosic substrate and the high ethanol yielding fusant strain was selected for the further study. A result and discussion on the above mentioned research work has been described in detail in this chapter.

5.1.2 Protoplast formation

The various biochemical and biophysical parameters such as enzyme concentration, lysis time and osmotic stabilizer has great influence on the formation of protoplasts. Therefore, the effects of the key parameters on the protoplast formation have been investigated and optimum conditions were established to achieve high yield of protoplasts.

Effect of lysing enzyme concentration

The lysing enzyme commonly used for the lysis of yeast cell wall is isolated from *Trichoderma harzianum*. The lysis of cell wall and formation of viable protoplasts greatly depends on the concentration of lysing enzyme. Therefore, the effect of lysing enzyme on the protoplast yield has been investigated at varying concentrations of enzyme in the range of 1-4 mg/mL. The protoplast yield was determined by counting the number of protoplasts released from the yeast cells. As indicated from Fig 6, the protoplast yield of *S. cerevisiae* was found to increase steadily with increase in enzyme concentration up to 2.5 mg/mL, remained almost constant till 3.5 mg/mL and decreased thereafter with further increase in enzyme concentration. A similar trend was also observed with the three xylose fermenting yeasts with maximum yield obtained at 3 mg/mL. The maximum yield of protoplasts was observed with *S. cerevisiae* (90.6%) followed by *P. tannophilus* (87%). The maximum protoplast yields with *P. stipitis* and *C. shehatae* were observed between 75 to 85%. In all the cases, the protoplast yield increased with increase in concentration up to a certain enzyme concentration and thereafter no significant impact was observed with higher enzyme concentration. The protoplast yield remained constant at high enzyme concentration due to limited site access and as a result there is no effect of the extra enzyme on the left over cell wall.

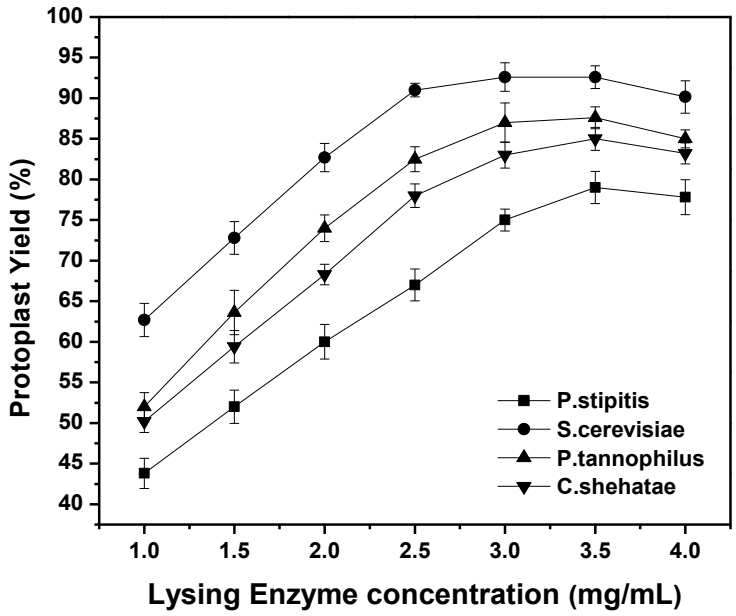


Fig. 6: Effect of lysing enzyme concentration on protoplast formation

Moreover, very high concentrations of enzyme were observed to be associated with the reduction in protoplast yield. This phenomenon may be due to the toxic effect caused by high enzyme concentrations. Similar observations were also made by Necas et al. and Balasubramanian et al. reporting that though increased concentration of lysing enzyme favors the protoplast formation, however, very high enzyme concentration may have toxic effect which is indicated by the occurrence of the lysis of protoplasts and thus reducing the protoplast yield [97, 98].

Effect of lysis time

Lysis time has a great impact on the protoplast formation as prolonged incubation may result in the damage of nascent protoplasts [99]. So, the effect of lysis time on protoplast formation was investigated by incubating yeast cells with lysing enzyme for varying time period of 1-3 h. The concentration of lysing enzyme was maintained at 3 mg/mL throughout the experiment. The number of protoplast released was monitored under phase contrast microscope and the results are depicted in fig 7. The numbers of protoplasts were observed to increase steadily with increase in lysis time up to 3 h in all cases and the protoplast yields were almost constant with further increase in lysis time.

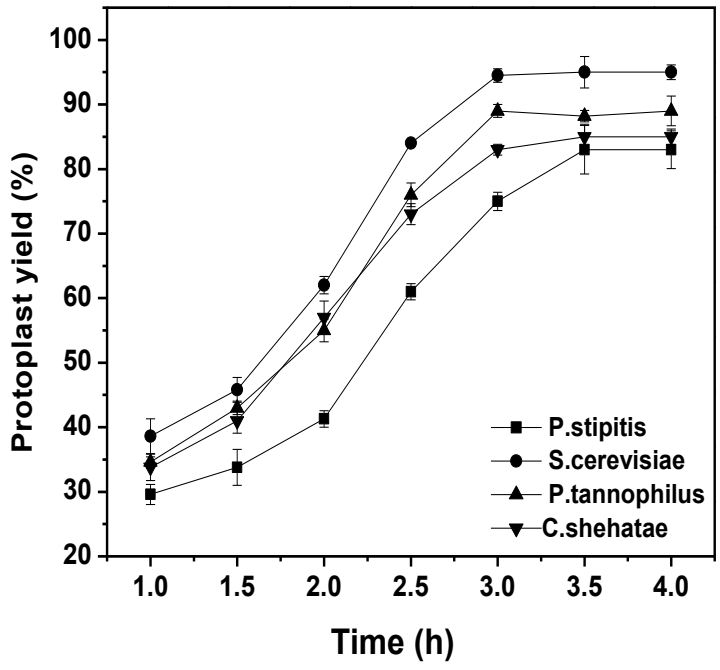


Fig. 7: Effect of lysis time on protoplast formation

The maximum yield of protoplast of *S. cerevisiae, P. tannophilus and C. shehatae* were found to be 95.3, 89.1 and 85.6% respectively. It is also observed that prolonged incubation beyond 3 h resulted in shrinkage of the protoplast. Hence the optimum lysis time for these strains has been established as 3 h. Though the similar trend was also observed with *P. stipitis*, the maximum yield of was obtained at higher lysis time (3.5 h). Furthermore, the variation in yield of protoplast among the yeast strains under study may be attributed to the variation in the thickness and complexity of cell wall of the strains.

Effect of osmotic stabilizers

The use of osmotic stabilizer is important to improve the stability of released protoplast leading to high yield of protoplasts as they support the protoplast from being lysed [100]. The osmotic stabilizers exerts external osmotic pressure to the protoplasts that substitutes for the hydrostatic pressure normally exerted by cell wall in intact cells. The different osmotic stabilizers such as KCl, sorbitol and $MgSO_4$ at 0.6 M concentration have been investigated to assess their effects on the protoplast formation.

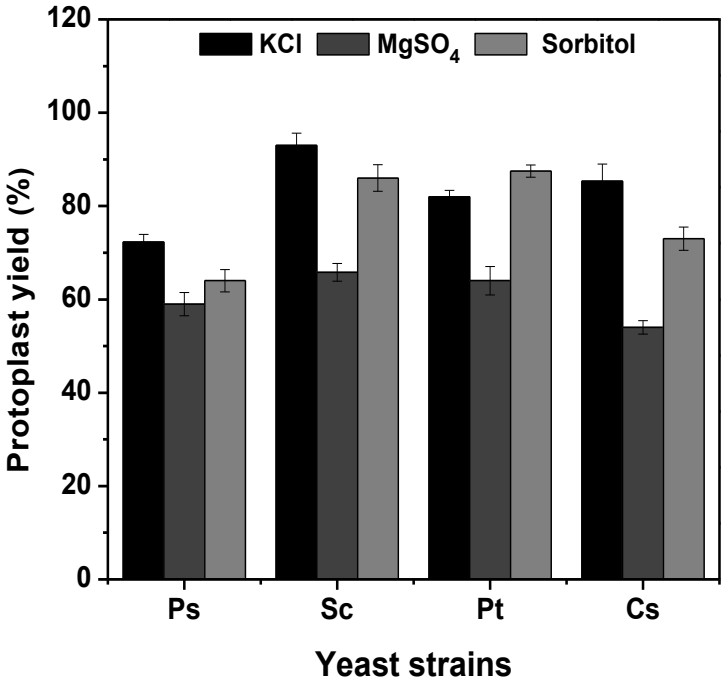


Fig. 8: Effect of various osmotic stabilizers on protoplast formation

As indicated from fig 8, among various osmotic stabilizers used under study, KCl is found to be most effective in releasing maximum number of protoplast from *S. cerevisiae, P. stipitis and C. shehatae.* The protoplasts formed were observed to be smaller in size using $MgSO_4$ and sorbitol. The maximum yield of protoplast released by *S. cerevisiae* is 95.7% followed by *C. shehatae* giving a yield of 86.5% using KCl as osmotic stabilizer. As per published literature, KCl is the reported to be the most preferred osmotic stabilizer for the formation of protoplast in yeasts [101- 103].

However, the exception was observed with *P. tannophilus* in which sorbitol was found to be the most effective osmotic stabilizer releasing maximum number of protoplasts. Further, in all cases the outer margin of protoplasts was observed to be irregular when the concentration of the osmotic stabilizer was deviated from 0.6 M in the range ± 0.2M. This phenomenon may be because of the imbalance in osmotic pressure around the protoplast.

SEM and phase contrast microscopy observation of protoplast formation

The effect of lysing enzyme on cell wall lysis was observed under both phase contrast microscope and scanning electron microscope (SEM). The figures 9 (a & b) show the cell wall lysis after 30 min and 60 min of incubation. The SEM observation of the lysis of yeast cell wall after 1 h of incubation showed the formation of fissures in the cell wall. The formation of fissures is attributed to the breakage of poly (1-3)-glucose bond of glucan by lysing enzyme [99]. Furthermore, the fissures were observed to be deepened and widened with increase in lysis time.

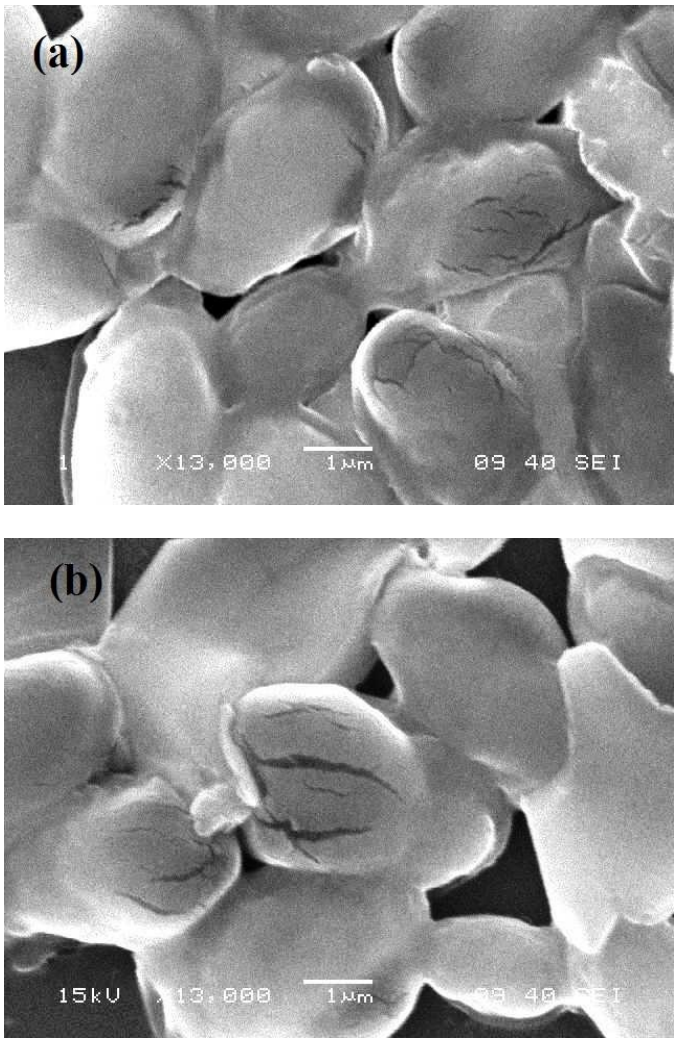


Fig. 9: SEM observation of cell wall lysis of *S. cerevisiae* after (a) 30 min (b) 60 min incubation with lysing enzyme

The observation of protoplast formation under phase contrast microscope indicates early breakage in cell wall of some cells before others. This may be due to the variation in the thickness of cell wall and age of cell. Fig 10 (a and b) shows the newly formed protoplasts (marked with arrow) while the other cells are still in the state of cell wall lysis. The protoplasts are completely spherical in shape which can be distinguished from the oval shaped yeast cells.

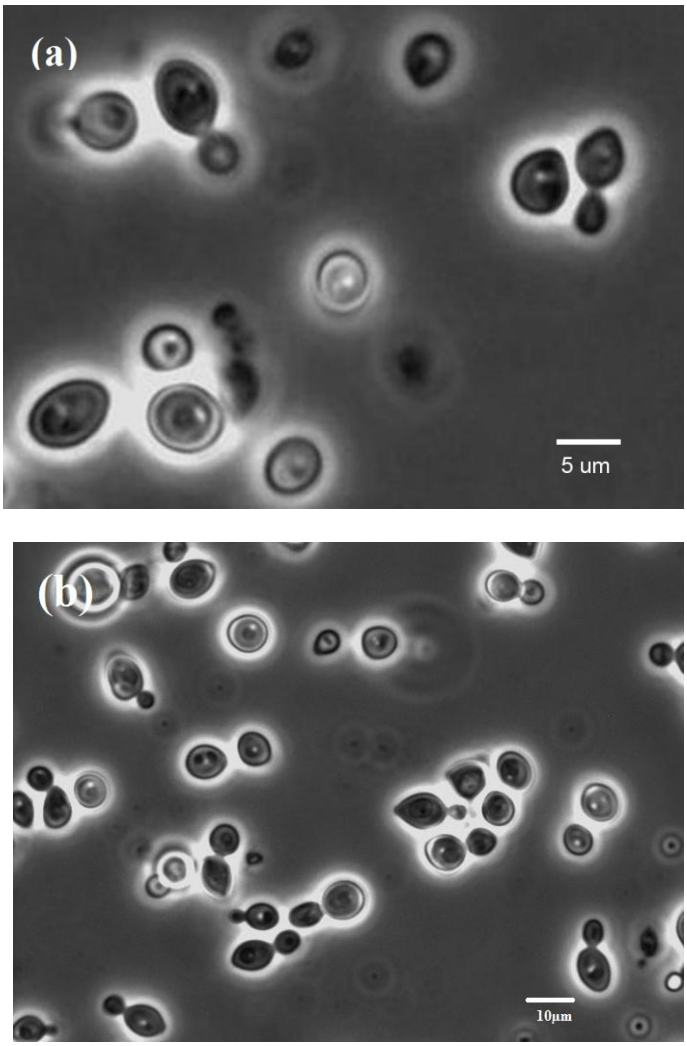


Fig. 10: (a) Release of protoplast observed under phase contrast microscope at a magnification of (a) 100X (b) 40X

5.1.3 Protoplast fusion

The protoplasts released by *S. cerevisiae* was fused with the protoplasts of three different xylose fermenting yeasts. The combinations of protoplast fusion were *S. cerevisiae* with *P. tannophilus (Sc + Pt)*, *S. cerevisiae* with *P. stipitis (Sc + Ps)* and *S. cerevisiae* with *C. shehatae (Sc + Cs).* The influence of key parameters for protoplast fusion was studied and optimum fusion conditions were established.

Effect of PEG concentration

The surface of isolated protoplasts carries negative charges (-10mV to -30mV) outside the plasma membrane and this leads to a strong tendency in protoplasts to repel each other due to same charges. The fusion of protoplast, therefore, needs some chemicals called fusogens to reduce their electronegativity and make them to fuse with each other. Chemical fusogen like polyethylene glycol (PEG) has been reported to facilitate the adherence of isolated protoplasts followed by fusion [27]. Furthermore, the concentration of the fusogen plays a vital role in the yield of maximum number of fused protoplast. The low concentration of PEG may not yield high frequency of fusants while the high concentration may be toxic to cells. Thus the optimization of the PEG concentration is of utmost importance. In this study, the concentrations of PEG in the range 25% to 45% were tested for the fusion of protoplast. Fig 11 indicates that the lower concentrations of PEG (<30%) do not stabilize the protoplasts because of the occurrence of swelling followed by rupture of protoplasts [28]. The maximum number of fused protoplasts was obtained using 35% PEG**.**

All the three combinations of yeast fusion showed very similar behavior in response to PEG concentration during protoplast fusion. Use of higher concentration of PEG above 35% did not show any favor to fusant formation. The higher concentrations of PEG had an adverse effect causing aggregation and over clumping of protoplast. Further, the fused protoplasts obtained with 40% PEG didn't grow well on the regeneration medium. This may be due to the toxic effect of use of high concentration of PEG causing damage to yeast protoplasts. Svoboda et al. also had a similar observation reporting that the high concentration of PEG is inefficient because of difficulty in rapidly and uniformly coating of viscous PEG solutions on protoplasts [104].

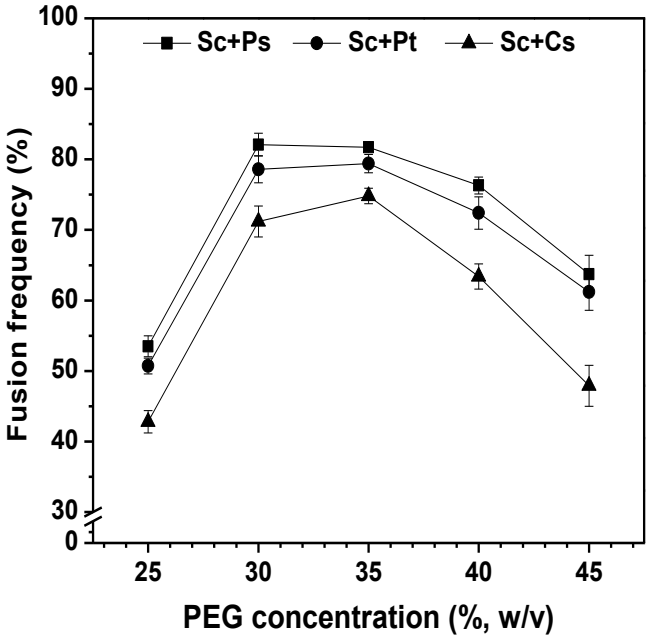


Fig. 11: Effect of PEG concentration on protoplast fusion

Effect of PEG exposure time

The effect of exposure time to PEG has a remarkable impact on the fusant formation as prolonged incubation with PEG may be toxic to protoplasts [28]. Hence, fusion time in the range of 10 to 40 min was investigated in this study to see its effect on the protoplast fusion. The range was selected based on the earlier reports on yeast protoplast fusion [4, 101, 105]. The experimental result is shown in fig 12. The results indicate that the exposure to PEG for less than 20 min was not sufficient time for the fusion of all the protoplasts. However, PEG treatment for 20 min showed maximum fusion frequency. Increase of PEG exposure time beyond 20 min resulted in the loss of viability of protoplasts that may be associated with the rupture of protoplasts due to dehydration. At 20 min of incubation in PEG solution, 82.1% of protoplasts of Sc + Ps combination were fused, while the value was 75.8% with Sc + Cs and 80.7 with Sc + Pt. Protoplasts incubated for more than 30 min in PEG did not grow well on regeneration medium. Similar observation of decrease in frequency of protoplast fusion and viable colony formation on prolonged incubation with PEG solution was also reported by Kao et al. [106].

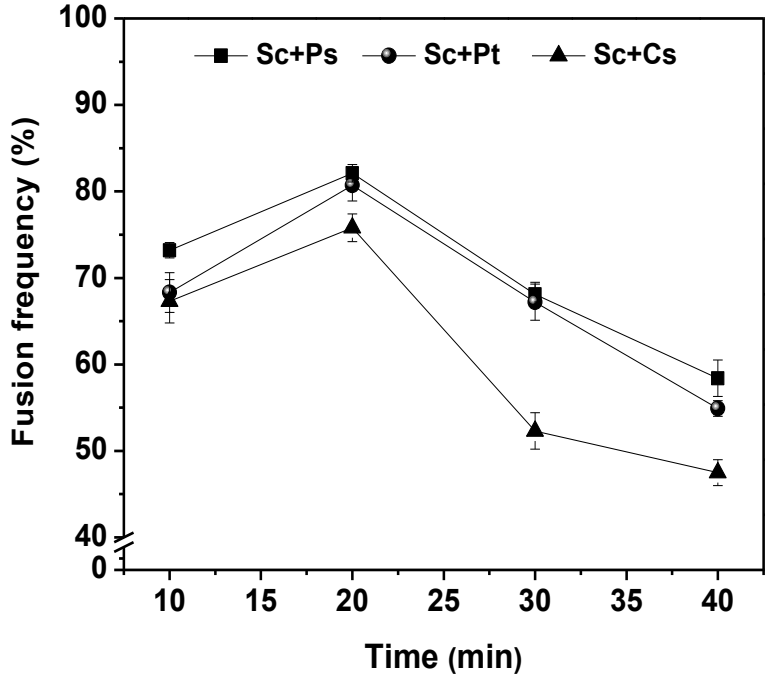


Fig. 12: Effect of PEG exposure time on protoplast fusion

Effect of pH

The change in pH may cause the change in the membrane permeability and enzyme activity of fused protoplast which may affect cell metabolism and ultimately the protoplast regeneration. Therefore, the effect of pH on protoplast fusion was investigated by carrying out protoplast fusion reaction at different pH. The pH range selected was between 5.5 and 8.5. It is observed that all the three combinations of fusion showed very similar behavior with varying pH values and all the fusant combinations showed maximum % protoplast fusion efficiency at 7.5 pH (Fig 13). At pH 7.5, the percentage protoplast fusion frequencies of all the three combinations of fusions were between 80 to 85%. Hence, pH 7.5 has been established as the optimum pH for the protoplast fusion under study.

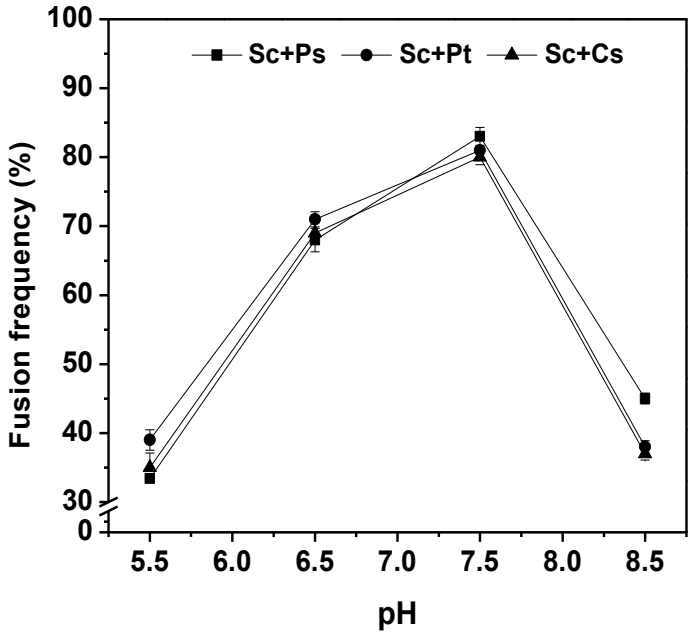


Fig. 13: Effect of pH on protoplast fusion

Phase contrast microscopy observation of protoplast fusion

The fusion of protoplasts was observed under phase contrast microscope and the images are presented in figure 14 (a & b). The lower magnification (40X), were used to count the number of protoplast fusing which was used to calculated fusion frequency, while the higher magnification (100X) was used to study the fusion of two protoplast in details. In the presence of PEG, the protoplasts were attracted and adhered to each other. The plasma membrane of two protoplasts disintegrated and the protoplasmic content fused, and ultimately two fused protoplast became one single and large sized protoplast.

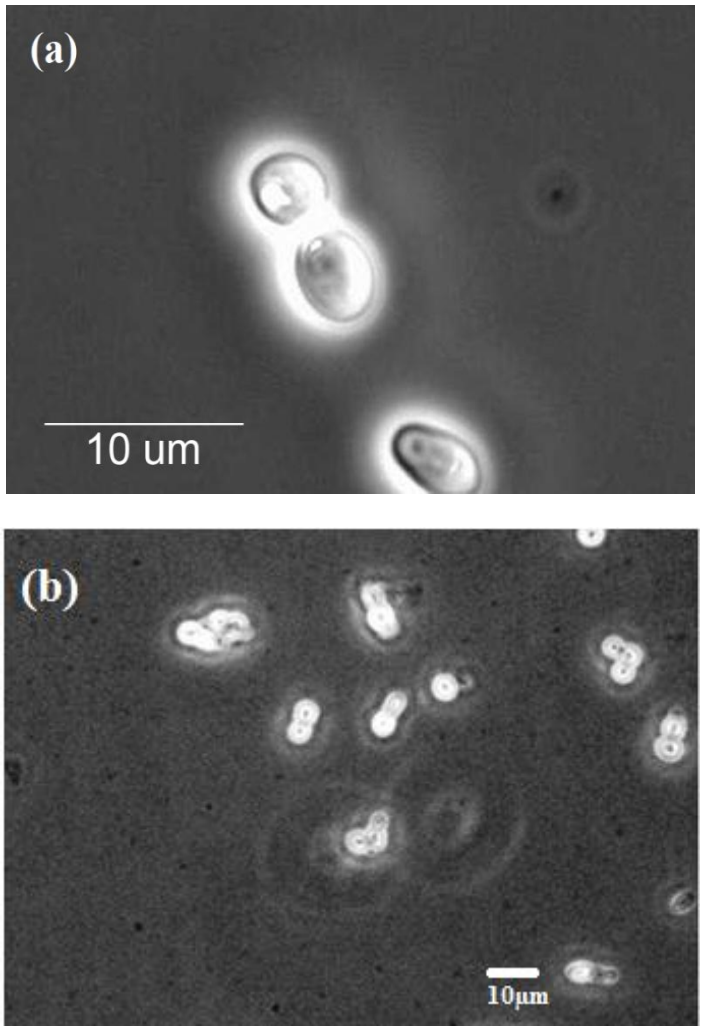


Fig. 14: Two protoplasts fusing under influence of PEG observed under phase contrast microscope at magnification of (a) 100X and (b) 40X 10µm

5.1.4 Sorting and regeneration of fusants

After fusion, the fusants were sorted by FACS and the results are represented as dot plots (Fig 15). The parental strains showed fluorescence in different gates based on the staining with fluorescent dyes FITC and R6G. Overlapping between fluorescence region of FITC and R6G was observed and possible compensation was done. As shown in figure 15, P4 is the sorting gate showing cells that exhibits high intensity of fluorescence for both FITC and R6G and thus are identified as fusants.

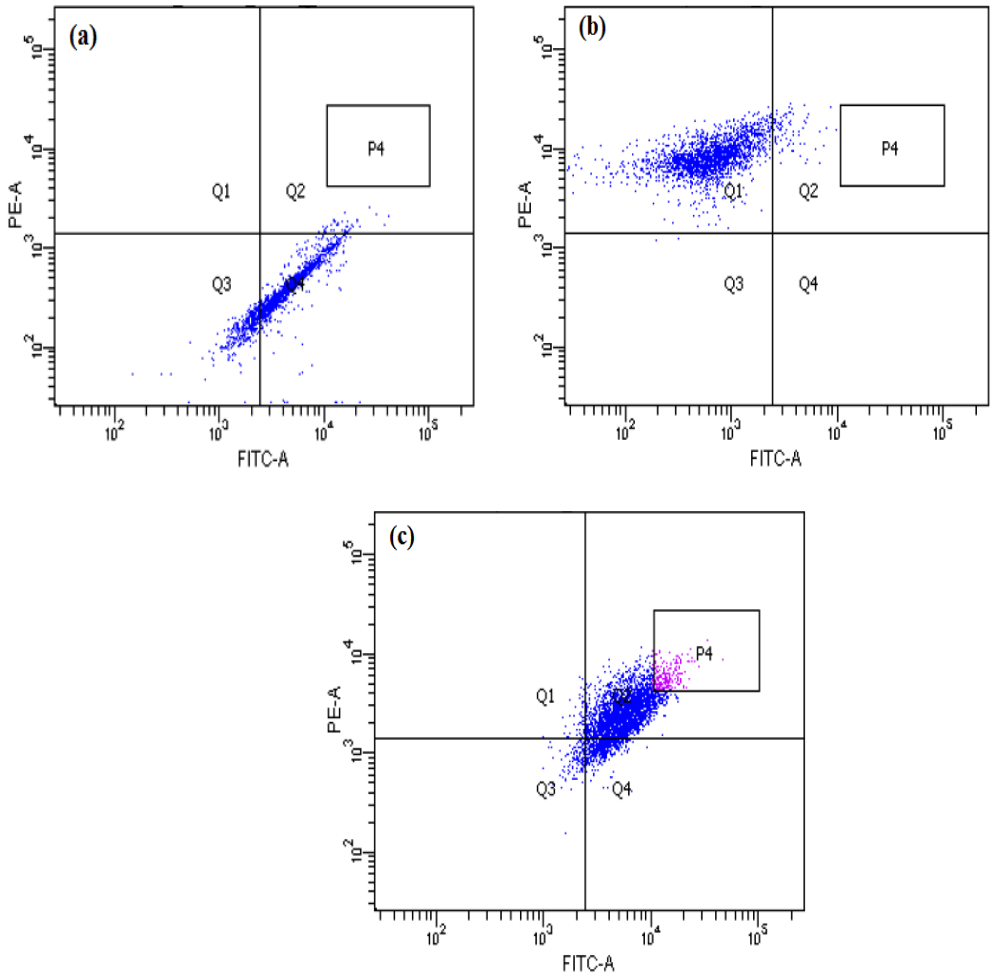


Fig. 15: Flow cytometric analysis of (a) Protoplasts of *S. cerevisiae* stained with FITC (b) Protoplasts of xylose fermenting yeast cell stained with R6G (c) Double positive fusants in P4 sorting gate exhibiting high intensity of fluorescence for FITC and R6G

About 12000 to 15000 fusants were sorted in 1h for each fusion and 300-500 colonies were found to survive which is evident from their growth on regeneration plates after incubation. The regenerated fusants showed rapid growth and colony formation. As shown in Fig 16, at an early stage of regeneration, the protoplasts were found to be of irregular shapes which may be due to the formation of discontinuous cell wall [97]. Out of 300 colonies grown on regeneration plate, only 70-95 colonies were able to grow on both YPD and YPX replica plates. The performances of these colonies were examined towards the bioethanol production.

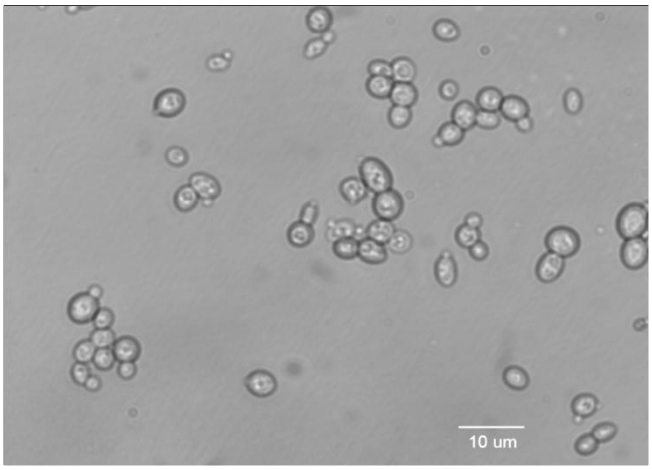


Fig. 16: Regeneration of protoplasts on regeneration medium

5.1.5. Evaluation of hybrid strains by ethanol fermentation

The performance of the hybrid strains towards ethanol production was examined by small scale fermentation experiment using a mixture of glucose and xylose (3:1 ratio). Table 4 presents the results of ethanol production by *S. cerevisiae* and the fusants that have shown high fermentation efficiency. Among the fusants, the fusant comprising of protoplasts of *S. cerevisiae* and *P. tannophilus* (RPR39 and RPR51) and *S. cerevisiae* and *P. stipitis* (RPR16 and RPR48) were able to produce ethanol with high efficiency. RPR51 and RPR16 have also shown comparable fermentation efficiency giving ethanol yield of 0.454 gg^{-1} and 0.437gg^{-1} respectively. These strains have been found to produce higher amount of ethanol compared to the ethanol produced by *S. cerevisiae* and other fusants. However, fusant RPR39 was found to be the most efficient strain producing maximum ethanol concentration (76.8 ± 0.31 gL^{-1}), ethanol productivity (1.06 gL^{-1}) and ethanol yield (0.458 $gL^{-1}h^{-1}$).

Ethanol yield was calculated as the grams of ethanol produced per gram of sugar consumed and ethanol productivity was calculated as the amount of ethanol (in grams per litre) produced per hour of fermentation.

Table 4: Ethanol production by *S. cerevisiae* and fusants using glucose-xylose mixture (3:1 ratio)

Organism	Fusion combination	Ethanol Conc. (gL^{-1})	Sugar consumed (gL^{-1})	Ethanol Yield (gg^{-1})[a]	Eth. Prod. ($gL^{-1}h^{-1}$)	Biomass (gL^{-1})
S.cerevisiae	-	69.1±0.57	153.8±0.76	0.436	0.96	6.98
RPR16	Sc+Ps	71.0±0.28	162.4±1.23	0.437	0.98	7.21
RPR39	Sc+Pt	76.8±0.31	167.5±0.82	0.458	1.06	7.32
RPR48	Sc+Ps	69.6±0.05	156.9±1.51	0.443	0.96	7.04
RPR51	Sc+Pt	73.9±0.97	162.7±1.47	0.454	1.02	7.28
RPR87	Sc+Pt	68.4±0.14	155.4±1.64	0.440	0.95	7.13
RPR119	Sc+Cs	63.4±0.14	146.6±1.14	0.432	0.88	7.08

Values are mean ± standard error of three determinations

[a] Mass of ethanol formed per mass of total sugar consumed

Fermentation of glucose-xylose mixture using RPR39

The study on time course of fermentation using highest ethanol yielding strain, RPR39 revealed that the strain consumed 98% of the glucose in the first 45 h of fermentation, and then xylose fermentation started (Fig 17). It has been reported that the hexoses are comparatively easier and faster assimilable substrates for ethanol production than pentose sugars [107]. Further, if fermentation time is not adequate, the pentoses remain unutilized in the medium thereby decreasing the overall rates of substrate utilization [107, 108]. It is observed that *S. cerevisiae* consumed only glucose without converting any xylose. The sugar conversion of 81.2 % was achieved with RPR39 which is higher than that of *S. cerevisiae* (77%). The rate of ethanol production increased steadily with time till 40 h of fermentation and then the rate was observed to be decreased. The high rate of ethanol production in the beginning of the fermentation is due to the fast consumption of glucose and then the rate decreased when xylose uptake started (Fig 17 and 18). Further, there was no increase in the concentration of ethanol after 60 h of fermentation using *S. cerevisiae* whereas the ethanol production continued till 72 h in

the case of RPR39. The ethanol yield obtained by RPR39 was 89.6 % of theoretical yield whereas for *S. cerevisiae* it was found to be 85.3 %. Figure 20 shows that the biomass of RPR39 was significantly higher than that of *S. cerevisiae* throughout the fermentation. The biomass production by RPR39 and *S. cerevisiae* were found to increase with time and the maximum biomass production was achieved at 36 h of fermentation. There is no further improvement in biomass production beyond 36 h of fermentation.

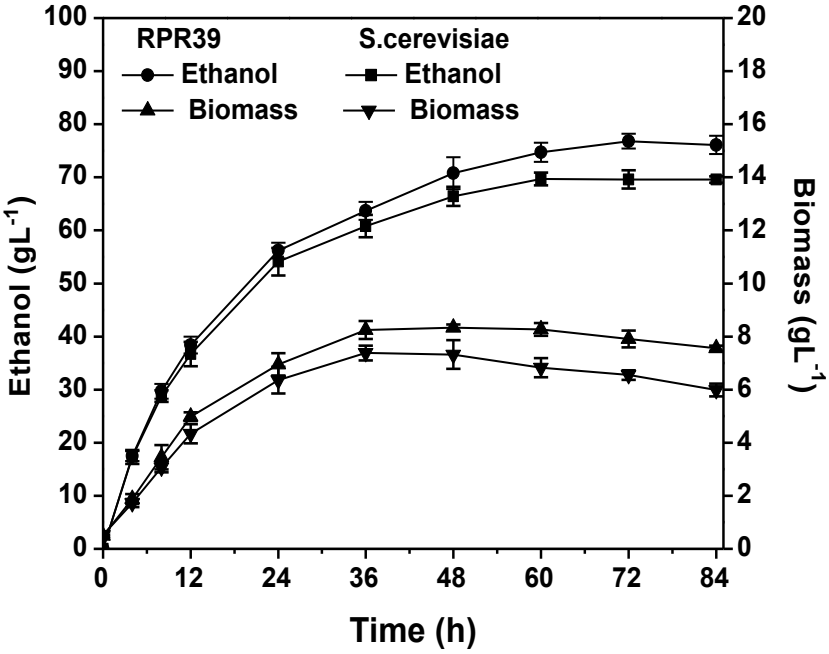


Fig. 17: Time course profiles of biomass and ethanol production during fermentation of 200 g L^{-1} of glucose-xylose mixture by *S. cerevisiae* and RPR39 fusant strain

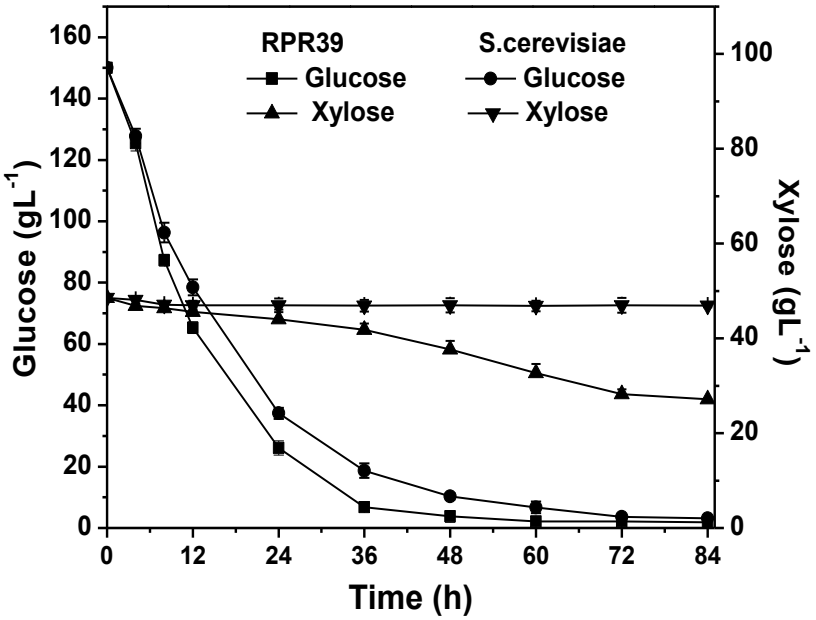


Fig. 18: Time course profiles of glucose and xylose concentration during fermentation of 200 g L^{-1} glucose- xylose mixture by *S. cerevisiae* and RPR39 fusant strain.

5.1.6 Stability study

It is an essential that the methods employed in the genetic manipulation of microorganisms must result in the formation of genetically stable products. The stability is most essential for the commercial use of the fusants for bioethanol production. So, the stability of the fusant was confirmed by assessing their substrate utilization and ethanol fermentation efficiencies using glucose-xylose mixture as substrate. Out of six fusants used under study, RPR51 and RPR48 were found to lose their stability and resembled to either of the parental strains after second passage of culturing, whereas RPR119 changed its substrate utilization properties within 3 months of regeneration. The other fusant strains RPR39, RPR87 and RPR16 were found to be stable and retained the fusant characteristics even after 9 months of subculturing. Furthermore, RPR39, the highest ethanol yielding fusant strain was found to be stable giving almost same amount of ethanol (i.e. 76.4 gL^{-1}, 75.94 $gL^{-1}$and 76.57 gL^{-1}) after 6, 12 and 18^{th} subculturing. RPR39 being the most efficient and a stable fusant strain was selected for further studies.

5.1.7 Genetic characterization of fusant

Relative DNA content of fusants

The study of the genotypes has indicated an increase in the DNA content of the fusant strain over the parental strains. The increased DNA content of the fusants is evident from the high PE width (PE-W) shown by the cell population at the P1 gate (Fig 19 a) whereas no such cell population was found in parental strains (Fig 19 b). Increased PE-W signifies the increased amount of DNA content in fusant and it is distributed in increased area, thus it takes more time to pass through the excitation focus. This population can be clearly differentiated from the 2N cells as they have just the double amount of DNA within a compact nucleus. From PE histogram represented in Fig 19 (d), it is clear that fusant DNA content (mean fluorescent intensity: 76.1±0.8) is nearly double to that of the unfused cells (mean fluorescent intensity: 39.4±1.7), which is in accordance with the observations made earlier by Mukai and Nakazawa et al. [81, 109].

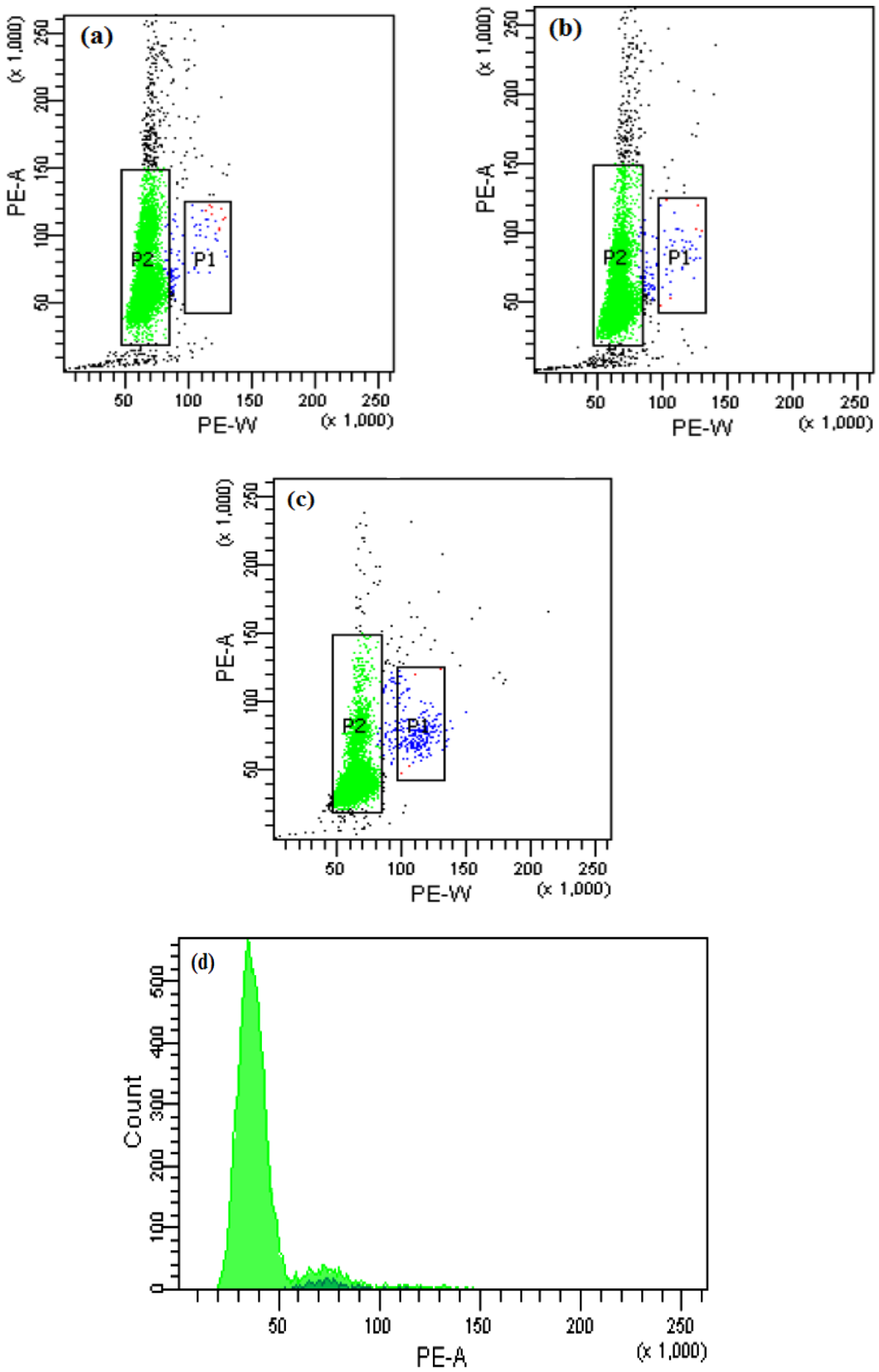


Fig. 19: Dot plot of PE-W/PE-A for DNA content of (a) *S. cerevisiae* (b) *P. tannophilus* (c) fusant and (d) PE-Area plot of fusant.

Random amplified polymorphic DNA

Molecular characterization of the fusant RPR39 was done to know the genetic differences and similarities of the fusant with parental strains. RAPD-PCR has frequently been used for identifying variations in DNA level among the yeast strains [105]. In the present study, the RAPD of the genome of fusant RPR39 and the two parental strains was done and the RAPD profile is shown in fig 20. The first and sixth lanes are the DNA ladders of 100 and 500 bp respectively. The four middle lanes represent the strains among which L1 and L2 represents RAPD profile of S. *cerevisiae* and *P. tannophilus* respectively. The profile of strain RPR39 represented in L4, shows a pattern of complementary bands between parental strains. This strain was observed to share some of the polymorphic bands (marked with arrows) with *S. cerevisiae* and some with *P. tannophilus*. Thus the RAPD profile justifies the fusant nature of the RPR39 strain. L3 is not the part of the present investigation.

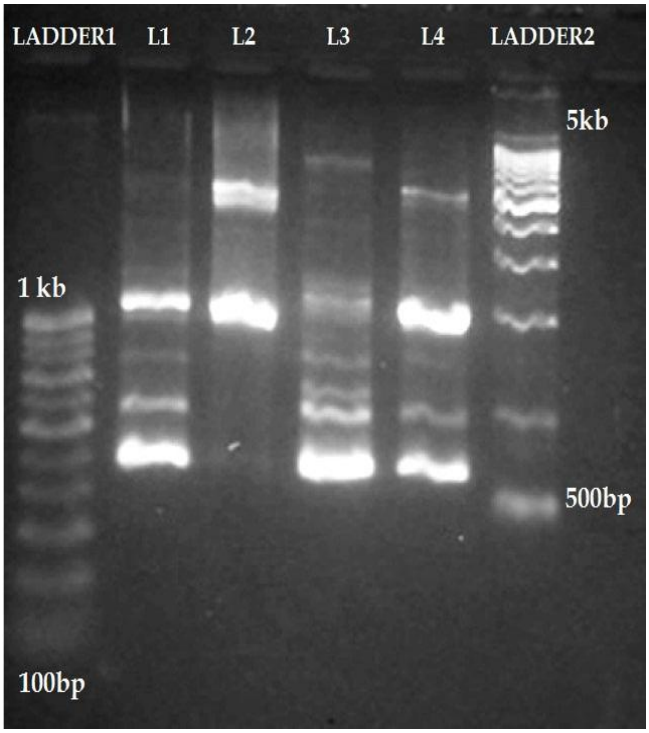


Fig. 20: RAPD profile of parental and RPR39 strain (Lane 1- SC, Lane 2- PT, Lane 4- RPR39 fusant).

DNA sequencing

The sequencing of a region of the r DNA gene unit, which includes two non-coding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene was performed for further confirmation of the fusant nature of RPR39 fusant. The ITS1 and ITS4 primers were used to amplify the ITS1 and ITS2 and 5.8S gene. The multiple sequence alignments of the sequenced region of RPR39 strain using CLUSTAL W showed 8 substitutions, 3 deletions and 1 insertion with respect to *S. cerevisiae* (NCIM -3090) which correspond to 95.38% sequence similarity whereas only 57.98% similarity was observed with *P. tannophillus* (NCIM-3502). Thus the genome of RPR39 is expected to consist of mainly the chromosomes of *S. cerevisiae* and shares only few genes with *P. tannophilus*. The results are in accordance with the findings of Yoon et al. and Selebano et al. as they reported that the fusant usually resembles any one of the parent in its properties [32, 110]. Kavanagh and Whittaker also supported the fact that the hybrids consist of the entire genome of the parent with which they shared most of the characteristics, together with a chromosome from the other parent [111].

The sequence of the ITS1, ITS2 and the 5.8S gene of the fusant RPR39 was submitted to Genbank with the NCBI ACESSION NO. JN887370. The multiple sequence alignment data of ITS 1, 5.8S and ITS2 regions of the parental strains *S. cerevisiae and P. tannophilu*s and the fusant RPR39 is shown in Fig 21. The colour codes for different nucleotide base shows the similarity in the sequence.

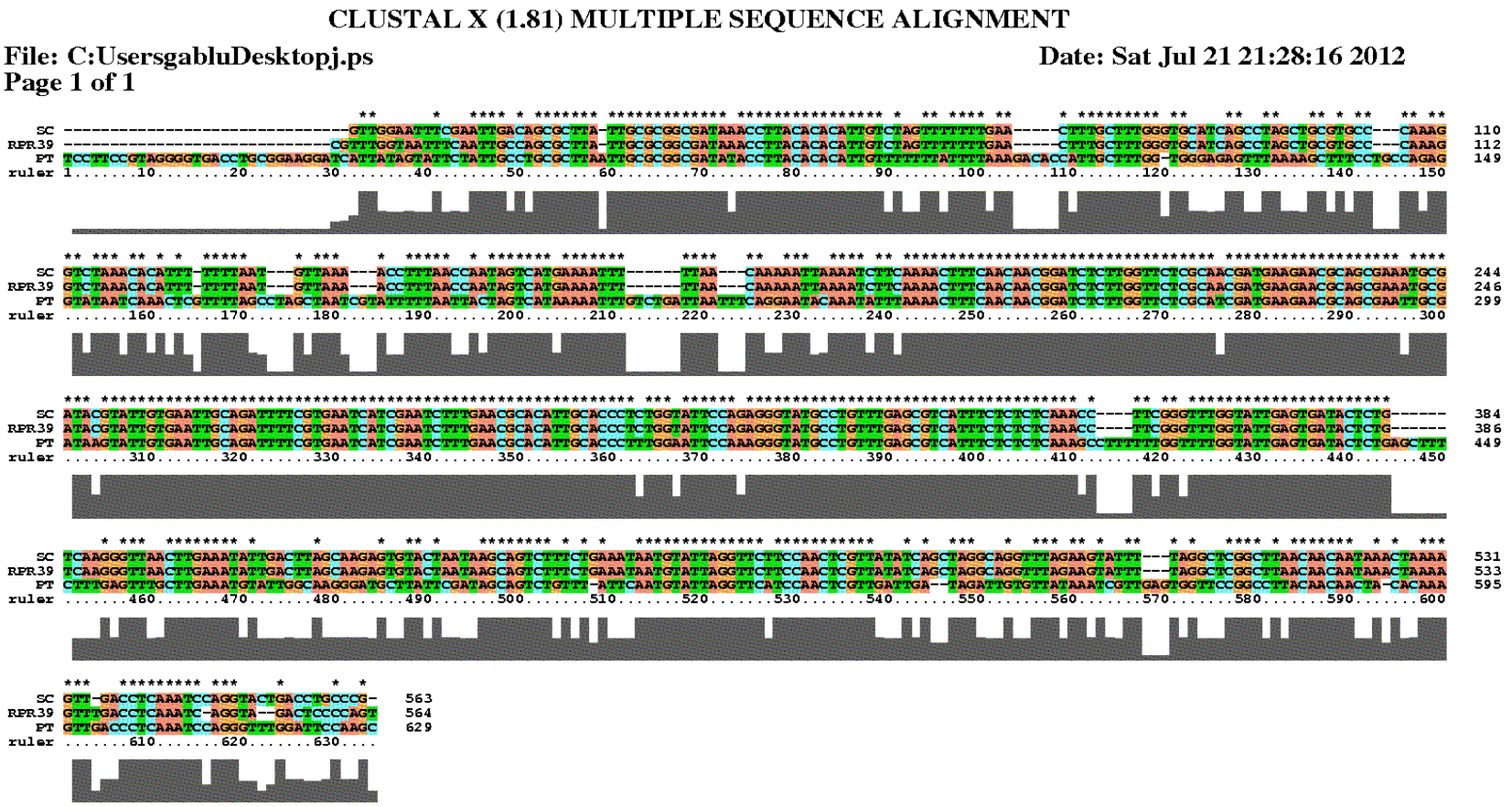


Fig. 21: Multiple sequence alignment of ITS1, ITS2 and 5.8s gene of fusant and parental yeast strains

(SC = *S. cerevisiae*, PT = *P. tannophilus*)

5.1.8 Comparison of the efficiency of fusant RPR39 with other yeast strains reported in published literature

A comparison of the efficiency of fusant RPR39 over other reported strains used for ethanol production from glucose-xylose mixture has been done (Table 5). It is indicated that there is a wide variation in ethanol yield in different studies. A study on development of recombinant yeast ScF2 by two rounds of genome shuffling reported the maximum ethanol yield of 0.4 gg^{-1} using mixture of glucose and xylose as model lignocellulosic hydrolysate [105]. A similar result of ethanol yield was also observed in another study reported by Zaldivar et al. using an industrial strain [95].

Various studies on co-culture of glucose and xylose fermenting yeasts for the fermentation of sugar mixture have been reported. Rouhollah reported an ethanol yield of 0.4 gg^{-1} using a co-culture of *S. cerevisiae* and *P. stipitis* and 0.36 gg^{-1} using a co-culture of *S. cerevisiae and P. stipitis* [112]. In another study Rivera et al. performed the fermentation of glucose-xylose mixture using a co-culture of *S. cerevisiae* and *P. stipitis* and reported the maximum ethanol yield of 0.40 gg-1 [113]. Recent, reports on fermentation of sugar mixture using protoplast fusants showed that the maximum ethanol yield achieved was 0.34 gg^{-1}. RPR39 has also shown much high fermentation efficiency as compared to the hybrid yeast strain used in other studies reported by Chmielewska and Yan et al. [33, 4].

Though an absolute comparison is difficult as the different studies were carried out under different fermentation conditions, the strain RPR39 developed in our study is found to be able to ferment both glucose and xylose efficiently giving higher ethanol yield as compared to most of the other reported values.

Table 5: A comparative study on ethanol production by various yeast strains using glucose-xylose mixture

Strain	**Description**	**Conditions**	**Carbon source**	**Ethanol produced (gL^{-1})**	**Ethanol Yield (gg^{-1})**	**Reference**
ScF2	Genome shuffling product	Fermentative	50 gL^{-1} glucose + 50 gL^{-1} xylose	40.0	0.40	[105]
A	Industrial strain	Anaerobic batch fermentation	50 gL^{-1} glucose + 50 gL^{-1} xylose	20.8	0.42	[95]
S. cerevisiae and P. stipitis	Co-culture	Fermentative	30 gL^{-1} glucose + 30 gL^{-1} xylose	29.45	0.41	[112]
P. stipitis and *K. marxianus*	Co-culture	Fermentative	30 gL^{-1} glucose + 30 gL^{-1} xylose	31.87	0.36	[112]
S. cerevisiae ITV01 and *P. stipitis* Y-7124	Co-culture	Aerobic batch fermentation	75 gL^{-1}glucose + 30 gL^{-1}xylose	30.3	0.40	[113]
Fusant 1	Protoplast fusant	Fermentative	30 gL^{-1}glucose + 20 gL^{-1}xylose	9.52	0.19	[4]
YD43-4	Protoplast Fusant	Fermentative	75 gL^{-1}glucose + 30 gL^{-1}xylose	-	0.348	[33]
S. cerevisiae RPR39	Protoplast Fusant	Fermentative	75 gL^{-1}glucose + 25 gL^{-1} xylose	76.8	0.458	This study

Part II

IMPROVEMENT OF HYBRID STRAIN BY MUTAGENESIS

5.2 DEVELOPMENT OF MUTANT HYBRID STRAIN

5.2.1 Introduction

Hybrid strains (fusants) normally have a tendency to segregate into parental strains. This instability limits the use of fusants industrially and further stabilization of RPR39 is obvious. Furthermore, during fermentation, yeast cells are subjected to multiple stresses that lead to adverse effect on the bioethanol yield and production. The stresses seem to have individual as well as synergistic effect on the viability and efficiency of yeast to produce ethanol [114]. It has been reported that, the ethanol stress reduces the cell metabolic activities and solute transport across the plasma membrane by increasing membrane fluidity whereas the thermal stress enhances the lag phase of yeast and affects the membrane functions [36]. Furthermore, the release of a variety of toxic inhibitors during the pretreatment of biomass also affects the fermentation performance. For example, phenolic compounds result in the loss of membrane integrity while organic acids and aldehyde inhibitors result in accumulation of reactive oxygen species [115, 116]. Therefore, besides the stability, the yeast strains used for ethanol fermentation of lignocellulosic substrates must have the important physiological properties like tolerance to high temperature, high ethanol concentration and toxic inhibitors. Hence, in this phase of dissertation work, efforts have been given for further improvement of the industrially important properties like genetic stability and stress tolerance of RPR39 hybrid strain by sequential mutagenesis. To the best of our knowledge, the present investigation is the first report where the protoplast fusant developed for simultaneous fermentation of hexoses and pentoses is sequentially mutated by multiple mutagens for the improvement of important characteristics.

The present chapter describes the details of the results and discussion on the mutagenesis of hybrid strain, stress tolerance study, evaluation of mutant for ethanol fermentation, its stability and genetic characterization. A comparison of the efficiency of mutant towards bioethanol production with other reported genetically manipulated strains was also included.

5.2.2 Mutagenesis and cell viability

It is reported that the stress tolerance in yeast strains is regulated by complex gene interactions [4]. Therefore, a number of methods have been the choice of researchers to improve the genetic constitution of industrially important microorganisms. Mutation is one of such method that can induce change in the genomic composition of any microorganism. In the present study, the time course study of mutagenic treatment using EMS, MNNG, near and far UV was performed to check the mutant cell viability before and after the mutagenesis. The results are represented in figure 22 and 23.

As depicted in fig 22, the percentage viability of cell suspension before mutagen treatment is found to be 92% and after 30 min of incubation the viability was reduced to 62% and 51 % using EMS and MNNG respectively. EMS was found to be less lethal to yeast cells than MNNG. The decrease in cell viability on exposure to MNNG for different time intervals has been shown in figure 22. The lethal effects of far and near ultraviolet light on mutations were also compared in figure 23. LD50 for far UV and near UV were 60 sec and 90 sec respectively.

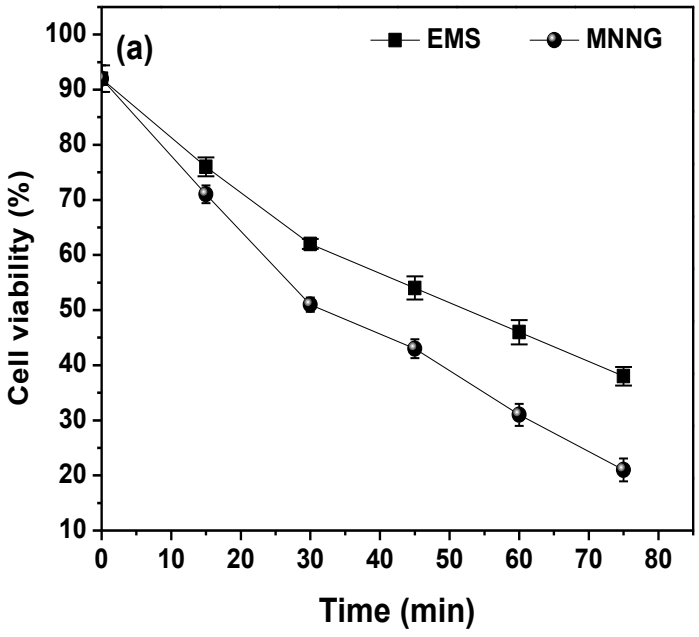


Figure 22: Effect of EMS and MNNG on percentage viability of mutant cells

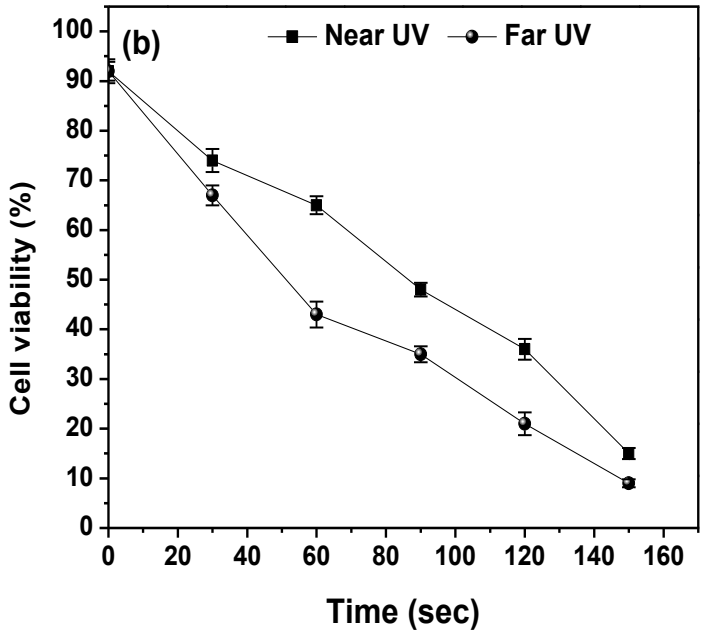


Figure 23: Effect of near UV and far UV on percentage viability of mutant cells

After 90 sec of exposure to near and far UV radiations, the percentage viability was reduced to 48 and 35 % respectively. Thus, yeast cells are found to be more sensitive to far UV than near UV radiations. Fig 24 shows the reduction in cell viability on exposure to MNNG for different time intervals. The figure shows that the exposure to MNNG is highly lethal to cells.

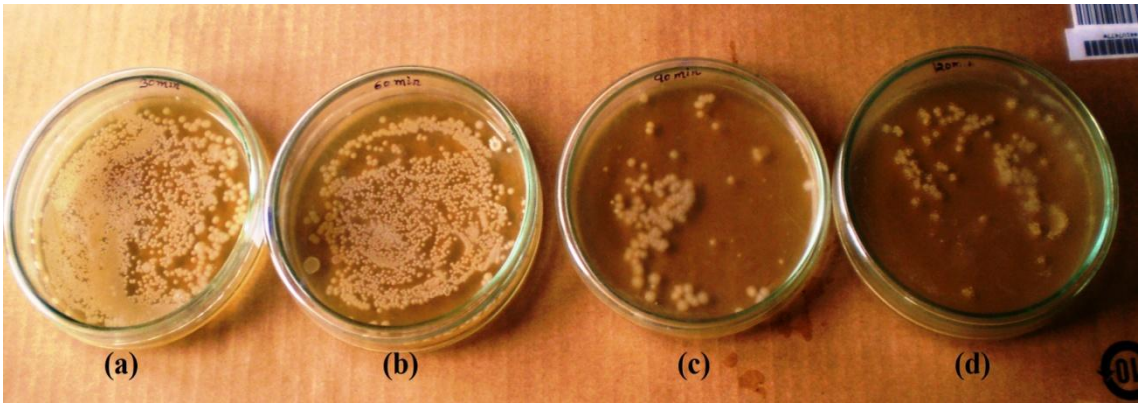


Fig. 24: Reduction in cell viability on exposure to MNNG for (a) 30 min, (b) 60 min, (c) 90 min and (d) 120 min

5.2.3 Stress tolerance study

Ethanol tolerance study

Among the various stresses encountered by yeast during ethanol fermentation, the most common stress is the increased ethanol concentration. High concentration of ethanol increases the membrane fluidity and destroys the membrane vital structures. In response to ethanol stress, yeast may change the content of membrane components such as unsaturated fatty acids and ergosterol and re-stabilize the membrane [86]. The development of ethanol tolerant yeast strain may be helpful for the production of high concentration of ethanol. Therefore, fermentation experiments were carried out at a varying ethanol concentration, in the range 6-10% (v/v) to assess the ethanol tolerance of developed mutant strains.

Among the several mutant yeast strains, the colonies of mutants that show improved tolerance to high ethanol concentration (≥ 6% initial ethanol) were selected. Table 6 represents the data of cell mass produced by the most stress tolerant mutant obtained from each type of mutagenic treatment. Among the eight mutants obtained from different mutagenic treatments, mut 3, 4, 7 and 8 showed better growth under stress of varying ethanol concentration. However, mut 7 showed the highest ethanol tolerance giving maximum biomass production at all level of ethanol concentrations under study. A substantial and comparable ethanol tolerance was also shown by mut 4 and 3.

The percentage cell mass produced by mut 3, 4 and 7 at 10% ethanol were 7.5%, 30% and 43.2% higher than RPR39, respectively. The mutants developed in this showed considerable ethanol tolerance till 7% initial ethanol but a severe decline in the biomass was observed with after initial ethanol concentration of 8%. This phenomenon may be attributed to the fact that ethanol at low concentrations inhibits cell division and decreases specific growth rate, while at high concentration it reduces cell viability and increases cell death [92].

Table 6: Cell mass produced by mutants using 175 gL^{-1} glucose-xylose under influence of ethanol stress

Strain name	Mutagenic treatment	Cell mass produced under stress conditions*				
		Initial ethanol % (v/v)				
		6	7	8	9	10
RPR39	None	51.5 ± 0.64	44.4 ± 0.75	36.4 ± 0.54	29.5 ± 1.53	16.4 ± 1.54
Mut1	EMS followed by Near UV	54.3 ± 0.82	46.5 ± 1.39	37.0 ± 2.04	26.4 ±0.43	14.6 ± 1.32
Mut2	EMS followed by Far UV	46.2 ± 0.25	40.6 ± 2.74	34.2 ± 1.25	29.4 ±1.47	16.4 ± 2.76
Mut3	MNNG followed by Near UV	57.0 ± 1.63	50.3 ± 1.46	43.2 ± 1.37	32.3 ±2.31	20.2 ± 1.53
Mut4	MNNG followed by Far UV	56.3 ± 1.84	49.5 ± 0.83	43.0 ± 0.35	33.0 ±1.34	21.3 ± 0.49
Mut5	Near UV followed by EMS	48.4 ± 0.63	42.7 ± 1.40	35.3 ± 0.62	27.8 ±1.74	14.3± 1.23
Mut6	Far UV followed by EMS	47.6 ± 2.13	44.2 ± 2.04	40.2 ± 1.48	30.9 ±1.53	18.2 ± 2.06
Mut7	Near UV followed by MNNG	61.8 ± 2.64	52.5 ± 1.55	46.2 ± 1.35	34.5 ±1.07	23.5 ± 1.73
Mut8	Far UV followed by MNNG	57.4 ± 0.96	47.2 ± 1.38	38.2 ± 0.14	27.6 ±0.53	19.3 ± 1.35

*The cell biomass values are expressed as percentage of control.

Thermotolerance study

Thermotolerant yeast helps in reducing cooling costs, distillation costs and has faster fermentation rates. These yeasts are also less prone to microbial contamination during fermentation [117]. The thermotolerance of mutants was evaluated by growing the yeasts in the temperature range 38^{o}C to 42^{o}C. The experimental results are shown in Table 7. The results show that among the mutants developed, mut 4 showed the highest thermotolerance, producing maximum amount of cell mass at the temperature range under study. A comparable thermotolerance was also observed with mut 1 and mut 7. The biomass production of 48.2%, 51.4% and 49.0% of control were obtained at 39^{o}C using mut 1, 4 and 7 respectively. Most of the mutants showed considerable amount of thermotolerance to 39^{o}C but further increase in temperature showed abrupt decline in the cell mass production.

Table 7: Cell mass produced by mutants using 175 gL^{-1} glucose-xylose under influence of thermal stress

Strain name	Mutagenic treatment	Cell mass produced under stress conditions*				
		Temperature (^{o}C)				
		38	39	40	41	42
RPR39	None	63.2 ± 1.43	39.6 ± 1.48	32.4 ± 1.43	20.0 ± 1.52	14.6 ± 0.36
Mut1	EMS followed by Near UV	68.2 ± 0.32	48.2 ± 1.64	37.4 ± 1.57	28.3 ±1.46	20.4± 0.84
Mut2	EMS followed by Far UV	60.2 ± 1.42	46.5 ± 1.32	30.4 ± 1.48	21.6 ±0.75	13.4 ± 1.36
Mut3	MNNG followed by Near UV	64.1 ± 1.84	47.9 ± 0.84	35.0 ± 2.71	25.7 ±1.52	18.8± 0.72
Mut4	MNNG followed by Far UV	72.4 ± 0.47	51.4 ± 2.04	44.2 ± 1.22	31.5 ±1.83	24.3± 0.68
Mut5	Near UV followed by EMS	56.3 ± 2.52	42.6 ± 1.52	29.4 ± 0.38	21.6 ±1.94	14.7 ± 1.15
Mut6	Far UV followed by EMS	60.0 ± 1.43	44.7 ± 1.74	33.4 ± 1.95	26.8 ±2.41	16.2± 0.61
Mut7	Near UV followed by MNNG	69.9 ± 1.58	49.0 ± 1.95	35.9 ± 1.86	28.5 ±0.99	19.4± 1.51
Mut8	Far UV followed by MNNG	53.5 ± 0.96	37.6 ± 1.66	28.0 ± 0.25	22.7 ±1.63	17.2 ± 0.75

*The cell biomass values are expressed as percentage of control.

Overall, among all the mutants developed, the maximum ethanol tolerance was observed in mut 7 while mut 4 exhibited highest thermal tolerance. A substantial thermotolerance was also observed in mut 7, while a significant and comparable ethanol tolerance was exhibited by mut 4. Further, compared to RPR39, mut 3 also showed significant tolerance to both stresses. Although mut 1 showed good thermotolerance, the strain was observed as less ethanol tolerant compared to mut 7. Thus, the strains 3, 4 and 7 were selected for further study on inhibitor tolerance.

Furthermore, a comparative study of various mutagenic treatments revealed that a combination of MNNG and UV is the most efficient mutagenic treatment in developing ethanol tolerant mutants. The mutant 7 showing high ethanol tolerance was developed using MNNG and near UV and mut 4 using MNNG and far UV as mutagens. The use of MNNG as potential mutagenic agent has also been reported earlier as it is associated with multiplicity in mutation and high frequency of mutants per survivor [41]. The development of improved thermotolerance [34] and ethanol tolerance [36] in yeast strains by use of UV radiations was also reported in earlier studies.

Inhibitor tolerance by mutants

The pretreatment of lignocellulosic biomass is considered as a prerequisite step for efficient saccharification and ethanol production. For example, the three major groups of by-product compounds produced during acid pretreatment of lignocellulosic biomass include sugar degradation products (furfural and hydroxyl-methyl furfural), lignin degradation products (phenolics compounds like vanillin) and compounds derived from lignocellulosic structure (acetic acid). It is evident from literature survey that the production of ethanol by yeast strains is severely inhibited by the generation of a number of toxic by-products such as phenolics, organic acids and furfural during the pretreatment process. Therefore, the development of novel yeast strains with increased tolerance toward fermentation inhibitors is highly desirable. With this aim, the mutants showing good tolerance to high temperature and ethanol (mut 3, 4 and 7) were selected for the assessment of their tolerance to various fermentation inhibiting molecules such as furfural, acetic acid and vanillin.

Tolerance to furfural

Furan compounds like furfural originates from Mailliard reactions of pentoses [118]. Furfural decreases the ethanol production and specific growth rate and gives rise to longer lag phase [115]. The mode of action involves the inhibition of central enzymes in glycolysis, like hexokinase, phosphofructokinase and triose phosphate dehydrogenase [119]. It also inhibits the activity of the enzymes involved in ethanol fermentation like alcohol dehydrogenase and aldehyde dehydrogenase [70]. Therefore the effect of furfural on the growth and ethanol fermentation by mutants were investigated and the experimental results are shown in Table 8.

Compared to the control, all the cultures supplemented with furfural showed a decrease in cell mass growth and ethanol production. The production of cell mass and ethanol by RPR39 and mut 4 were observed to be severely affected by the presence of furfural. Compared to mut 7, mut 3 showed higher tolerance to low concentration of furfural whereas at high concentrations (1-2 gL^{-1}), mut 7 showed better activity producing more cell mass and ethanol. At the initial furfural concentration of 2 gL^{-1}, ethanol produced by mut 3 and 7 were 29.5 and 44.1 % higher than RPR39. The ability of yeast to tolerate furfural is directly coupled to the ability of converting furfural to less inhibitory compound like furfuryl alcohol [120]. In the present study, mut 7 is shown to have significant ability to tolerate high concentration of furfural.

Tolerance to vanillin

Vanillin is produced due to degradation of lignin and their amount depends on the lignin content of biomass. Vanillin is reported to have adverse effect on the biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barrier and enzyme matrices [121]. Therefore the effect of vanillin on the growth and ethanol fermentation by mutants were studied and the results are shown in Table 9.

As indicated in table 9, mut 3 exhibited highest tolerance to at all concentrations of vanillin followed by mut 4. The sensitivity towards vanillin was higher in case of mut 7 as compared to mut 3 and 4. At 1 gL^{-1} vanillin in fermentation medium, these two mutants produced 13.6 gL^{-1} and 12.1 gL^{-1} ethanol respectively which is 38.7 % and 23.4 % higher than ethanol produced by RPR39.

Table 8: Cell mass and ethanol produced by mutants and fusant RPR39 using 175 gL^{-1} glucose-xylose in presence of furfural

	Conc. (gL^{-1})	RPR39 (fusant)		Mut 3		Mut 4		Mut 7	
		Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})
Control	0	100	65.6 ±1.3	100	73.2 ± 2.5	100	69.1 ± 3.1	100	73.6 ± 2.7
Furfural	0.25	73.5 ± 4.9	45.3 ± 1.6	78.9 ± 1.5	51.2 ± 1.6	67.8 ± 2.4	38.4 ± 1.4	78.0 ± 2.4	54.4 ± 2.5
	0.5	53.5 ± 3.8	38.4 ± 0.9	60.5 ± 2.6	40.4 ± 1.0	50.1 ± 1.4	29.4 ± 0.5	58.2 ± 2.0	43.5 ± 1.9
	1	30.4 ± 1.5	26.2 ± 1.4	38.7 ± 3.8	26.8 ± 0.8	24.6 ± 1.7	22.0 ± 1.8	40.8 ± 2.5	32.6 ± 2.6
	2	18.6 ± 2.7	14.2 ± 1.5	22.4 ± 1.6	18.4 ± 1.3	17.2 ± 1.8	13.7 ± 0.9	23.4 ± 2.2	20.6 ± 0.4

***The results of biomass are expressed as percentage of control**

Table 9: Cell mass and ethanol produced by mutants and fusant RPR39 using 175 gL^{-1} glucose-xylose in presence of vanillin

	Conc. (gL^{-1})	RPR39 (fusant)		Mut 3		Mut 4		Mut 7	
		Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})
Control	0	100	65.6 ±1.3	100	73.2 ± 2.5	100	69.1 ± 3.1	100	73.6 ± 2.7
Vanillin	0.25	44.6 ± 1.6	32.4 ± 1.5	54.2 ± 1.4	45.3 ± 0.2	48.1 ± 2.0	44.2 ± 1.4	47.5 ± 1.7	38.2 ± 1.3
	0.5	40.4 ± 1.2	26.0 ± 0.7	46.2 ± 2.1	38.3 ± 1.3	44.6 ± 0.2	39.3 ± 1.5	42.3 ± 0.5	32.4 ± 2.0
	0.75	16.1 ± 0.7	11.9 ± 1.4	23.1 ± 1.1	26.3 ± 0.9	20.5 ± 1.7	25.6 ± 0.7	19.4 ± 1.5	21.4 ± 1.4
	1	9.9 ± 1.6	9.8 ± 1.8	11.6 ± 1.9	13.6 ± 1.6	8.8 ± 1.5	12.1± 1.5	8.5 ± 2.0	10.4 ± 0.5

***The results of biomass are expressed as percentage of control.**

Furthermore, among all the inhibitors tested, vanillin showed highest inhibitory effect on both ethanol production and biomass growth. The high toxicity of vanillin in comparison to acetic acid and furfural was also reported by Delegenes et al. They reported that both biomass growth and ethanol production processes in xylose fermenting yeasts were almost completely inhibited at an initial vanillin concentration of 1.0 gL^{-1} [122].

Tolerance to acetic acid

Acetic acid is formed by the de-acetylation of hemicelluloses and inhibits ethanol fermentation by reducing biomass formation and ethanol yields. The acetic acid has been reported to be associated with the uncoupling and intracellular anion accumulation. It decreases the intracellular pH and decrease in pH is compensated by the plasma membrane ATPase, thereby reducing the availability of ATPs towards biomass formation [123]. Therefore the influence of acetic acid on the growth and ethanol fermentation by mutants were studied and results are shown in Table 10.

In comparison to vanillin and furfural, acetic acid has shown to have less toxic effect on all the mutant strains used under study. Among the various mutants, mut 7 showed the highest tolerance to all the concentrations of acetic acid while the intensity of inhibition was highly distinguished in mut 4 and fusant RPR39 (Table 10). With an acetate concentration of 2 gL^{-1}, the ethanol produced by the fusant RPR39 was 54.2 gL^{-1} whereas with addition of 8 gL^{-1} of acetic acid it was reduced to 35.5 gL^{-1}.

Overall, mut 3 was found to exhibit highest tolerant to vanillin, while mut 7 showed highest tolerance to acetic acid and high concentrations of furfural. Considering the inhibiting effects of all stress factors under study, mut 7 was found to be the most stress tolerant strain exhibiting improved tolerance to high initial ethanol concentration and fermentation inhibitors like furfural and acetic acid. Moreover, the strain has shown a comparable growth at 39^{o}C and 40^{o}C with respect to the fusant RPR39 though a slight decrease in growth was observed at 42^{o}C. Therefore, mut 7 was established as the most stress tolerant strain in this study.

Table 10: Cell mass and ethanol produced by mutants and fusant RPR39 using 175 gL^{-1} glucose-xylose in presence of acetic acid

	Conc. (gL^{-1})	RPR39 (fusant)		Mut 3		Mut 4		Mut 7	
		Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})	Biomass*	Ethanol (gL^{-1})
Control	0	100	65.6 ±1.3	100	73.2 ± 2.5	100	69.1 ± 3.1	100	73.6 ± 2.7
Acetic acid	2	68.3 ± 1.5	54.2 ± 1.6	73.5 ± 1.8	62.7 ± 1.3	66.3 ± 1.7	61.3 ± 1.8	75.6 ± 2.4	66.7 ± 1.5
	4	62.4 ± 2.1	46.3 ± 1.3	69.2 ± 1.3	54.7 ± 1.1	63.2 ± 1.1	53.2 ± 1.9	72.3 ± 3.1	58.4 ± 0.6
	6	53.2 ± 1.5	38.3 ± 2.2	58.3 ± 2.0	45.4 ± 2.1	54.6 ± 0.9	46.3 ± 0.3	64.2 ± 1.4	49.5 ± 1.2
	8	45.2 ± 1.3	35.5 ± 1.3	52.4 ± 1.6	41.3 ± 2.9	45.3 ± 2.0	38.2 ± 1.7	55.2 ± 2.0	46.4 ± 1.5

***The results of biomass are expressed as percentage of control**

Ergosterol and trehalose assay

The stored ergosterol and trehalose content in selected mutant strains were determined as these two factors are reported to be associated with the stress tolerance in yeasts. Ergosterol regulates the balance among membrane components such as lipids and proteins and thus plays a critical role in ethanol resistance in *S. cerevisiae* [86]. As shown in figure 25 (a), the ergosterol content of all of the selected mutants is higher than RPR39 though observed with varying degree of expression level. The high ergosterol content was observed in mut 3 and 7 compared the fusant RPR39.

It is evident from literature that, the stored trehalose content in yeast cells provides higher resistance to stress conditions such as high ethanol concentration and osmotic pressure by preserving the integrity of biological membranes and suppressing the aggregation of denatured proteins [124]. It has also been reported that during ethanol stress, trehalose functions as a chemical co-chaperone and prevents the aggregation of the misfolded proteins on the membrane [125]. In the present study, the trehalose content of mutant strain mut 4 and 7 were higher than the trehalose content of RPR39 (Fig 25 b). The mut 7 shows the highest trehalose content. The high trehalose and ergosterol accumulation in mut 7 seem to contribute membrane stabilizing effects and ultimately to the increased thermotolerance and ethanol tolerance. Thus, trehalose accumulation was found to be related to the ethanol tolerance and thermotolerance of the mutants. This is in good agreement with the findings of Ogawa et al. and Singer et al. [125]. The accumulation of trehalose and ergosterol under the effect of heat shock has also been confirmed by Swan and Watson [86].

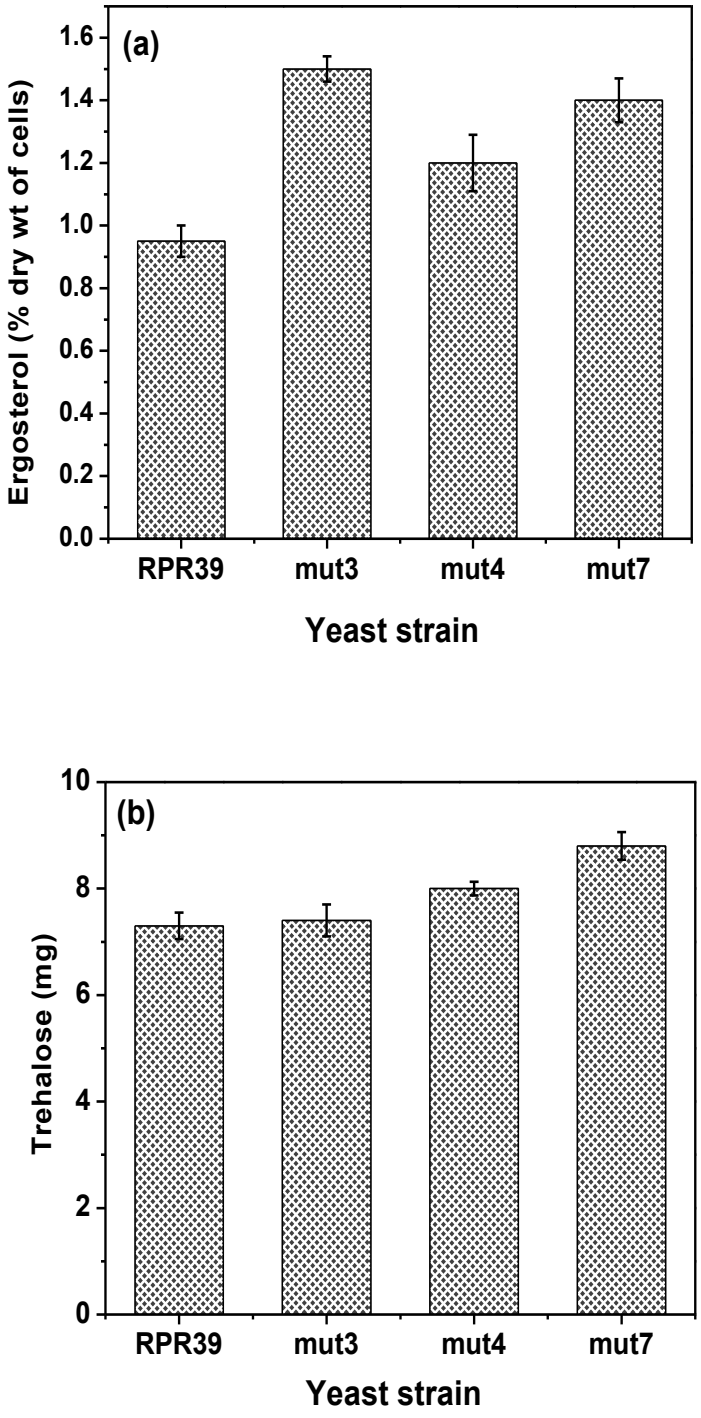


Figure. 25: (a) Ergosterol and (b) Trehalose content of yeast mutants and RPR39 during ethanol fermentation of glucose-xylose mixture.

5.2.4 Ethanol production by mutants under normal conditions

The performance of mutants towards bioethanol production was evaluated by small-scale fermentation experiments using glucose-xylose mixture as substrates. Among the mutants, the highest ethanol production was achieved using mut 7 producing 16.6 % higher than RPR39 (65.6 gL^{-1}) as shown in figure 26 (a). Comparable ethanol productions were also achieved by mut 1 and mut 3 while a negative effect of mutagenesis on the ethanol production has been observed with mut 2, 5 and 8 producing 45.8 gL^{-1}, 54.4 gL^{-1} and 56.9 gL^{-1} ethanol respectively. Fig 26 (b) shows that the cell mass produced by the strains during fermentation was directly related to their ethanol production efficiencies. The maximum cell mass was produced by mut 7 (7.21 gL^{-1}) followed by mut 1 and 3.

The study of fermentation using mut 7 shows that the mutant produced 73.6 gL^{-1} ethanol from 155.3 gL^{-1} glucose-xylose mixture. The corresponding ethanol yield, productivity and sugar conversion were calculated as 0.461 gg^{-1}, 1.05 $gL^{-1}\,h^{-1}$ and 86.2 % respectively.

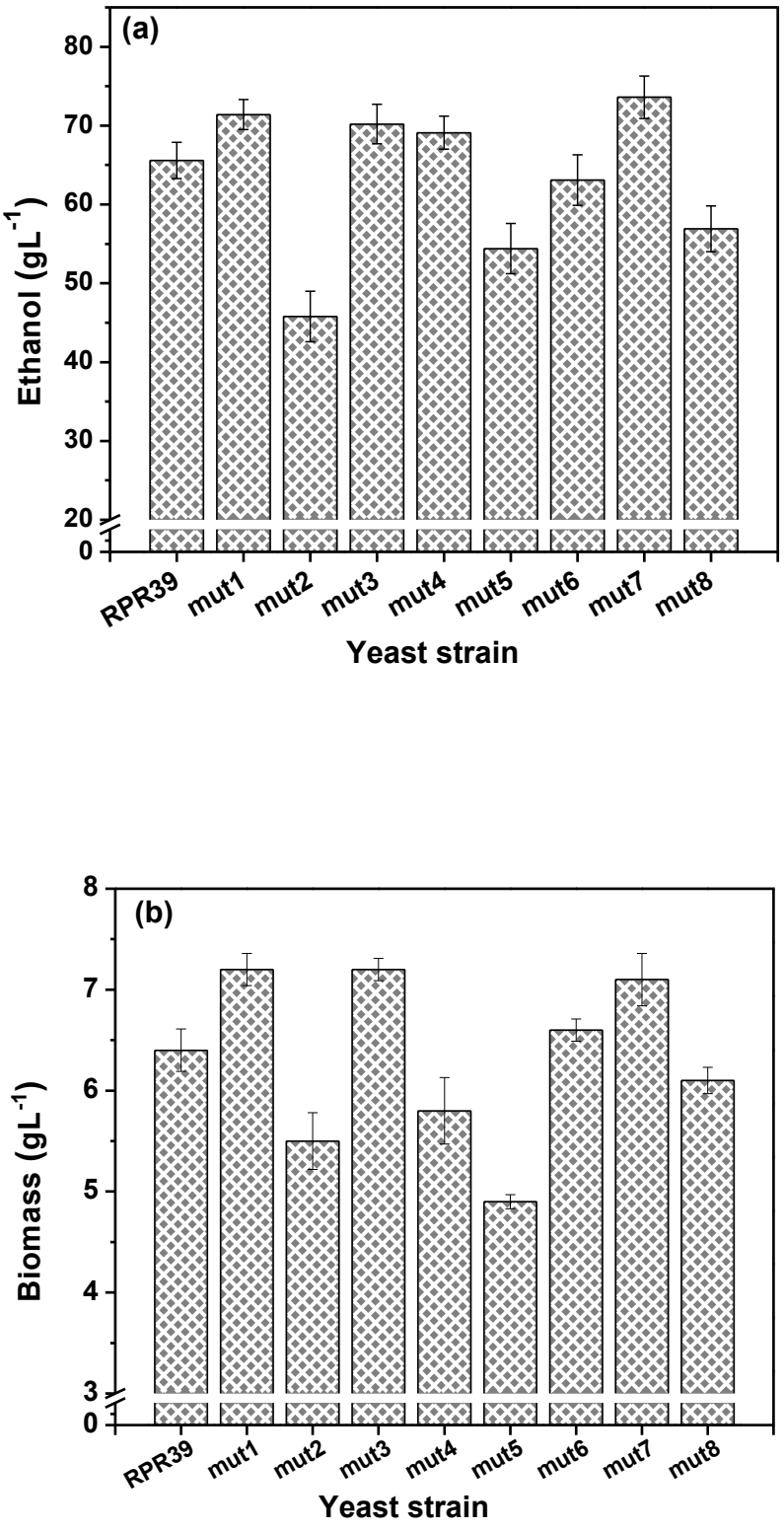


Fig. 26: (a) Ethanol and (b) cell mass produced by mutants and fusant RPR39 during ethanol fermentation using glucose-xylose mixture.

A comparison of the ethanol production by mut 7 with the other recombinant strains using glucose-xylose mixture under fermentative condition was done. Although an absolute comparison is not possible because of variation in glucose-xylose ratio and fermentation conditions in the other reported studies, the overall comparison showed that the ethanol yield of RPRT90 is either high or comparable to the ethanol yield reported by others [15, 126, 127].

In a study reported on fermentation of mixed sugars using recombinant yeast strain *Saccharomyces* 1400 (pLNH33), the authors reported an ethanol yield of 0.46 gg^{-1} during fermentation of glucose-xylose mixture under normal fermentation conditions [15] while a recombinant strain MA-R4 produced ethanol with an yield of 0.42 gg^{-1} [5]. The, ethanol yield achieved using RPRT90 (0.461 gg^{-1}) is higher than both of these recombinant strain. Thus RPRT90 can be considered as an efficient strain co-fermenting glucose and xylose simultaneously.

5.2.5 Stability of mutants

The ethanol yield and stress tolerance of all the 8 mutants were assessed up to 12 generations (9 months). Among the eight mutants obtained, mut 1, 7 and 4 were found to be stable till 9 months of subculturing while mut 3, 5 and 8 lost their stability in either stress tolerance properties or ethanol production efficiency within third subculturing. The thermotolerance property of mut 2 and 6 was found to be reduced after 4 months of subculturing. Mut7, the mutant showing highest tolerance towards various stresses was found to be stable in its tolerance properties and it maintained all of its features even after 9 months. The ethanol produced by mut 7 after first, fifth and tenth subculturing was 73.4 gL^{-1}, 73.6 gL^{-1} and 73.1 gL^{-1} respectively. Thus, it is evident that mut 7 is the most efficient strain found in respect to both stress tolerance and genetic stability. Mut 7 was later designated as **RPRT90.**

5.2.6 Genetic characterization of mutant

Molecular characterization of the mutant and the fusant affirmed that the mutation has brought profound change in the genetic composition of the mutant. The RAPD profile of RPRT90 strain displayed the presence of monomorphic bands which were absent in fusant RPR39 (Fig 27).

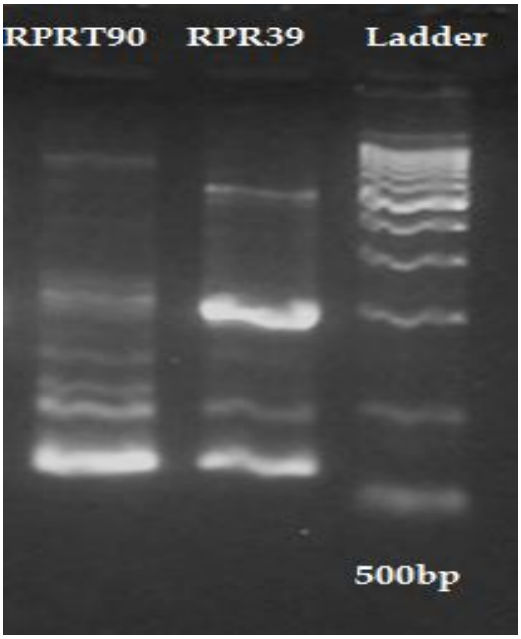


Fig. 27: RAPD profile of fusant RPR39 and mutant RPRT90

Further, characterization of mutant was done by partial sequencing of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene of mutant RPRT90 and fusant RPR39 genomes. The multiple sequence alignments of sequenced regions of RPRT90 strain (NCBI Accession No. JN887371) showed 7 substitutions, 2 deletions and 2 insertions with respect to RPR39 strain (NCBI Accession No. JN887370) which correspond to 95.34% sequence similarity. The presence of monomorphic bands in the RAPD profile and the difference in nucleotide sequence (4.66 %) in multiple sequence alignment data confims the taxonomic separation of the mutant from the fusant RPR39. The multiple sequence alignment data of the fusant RPR39 and mutant RPRT90 is presented in figure 28.

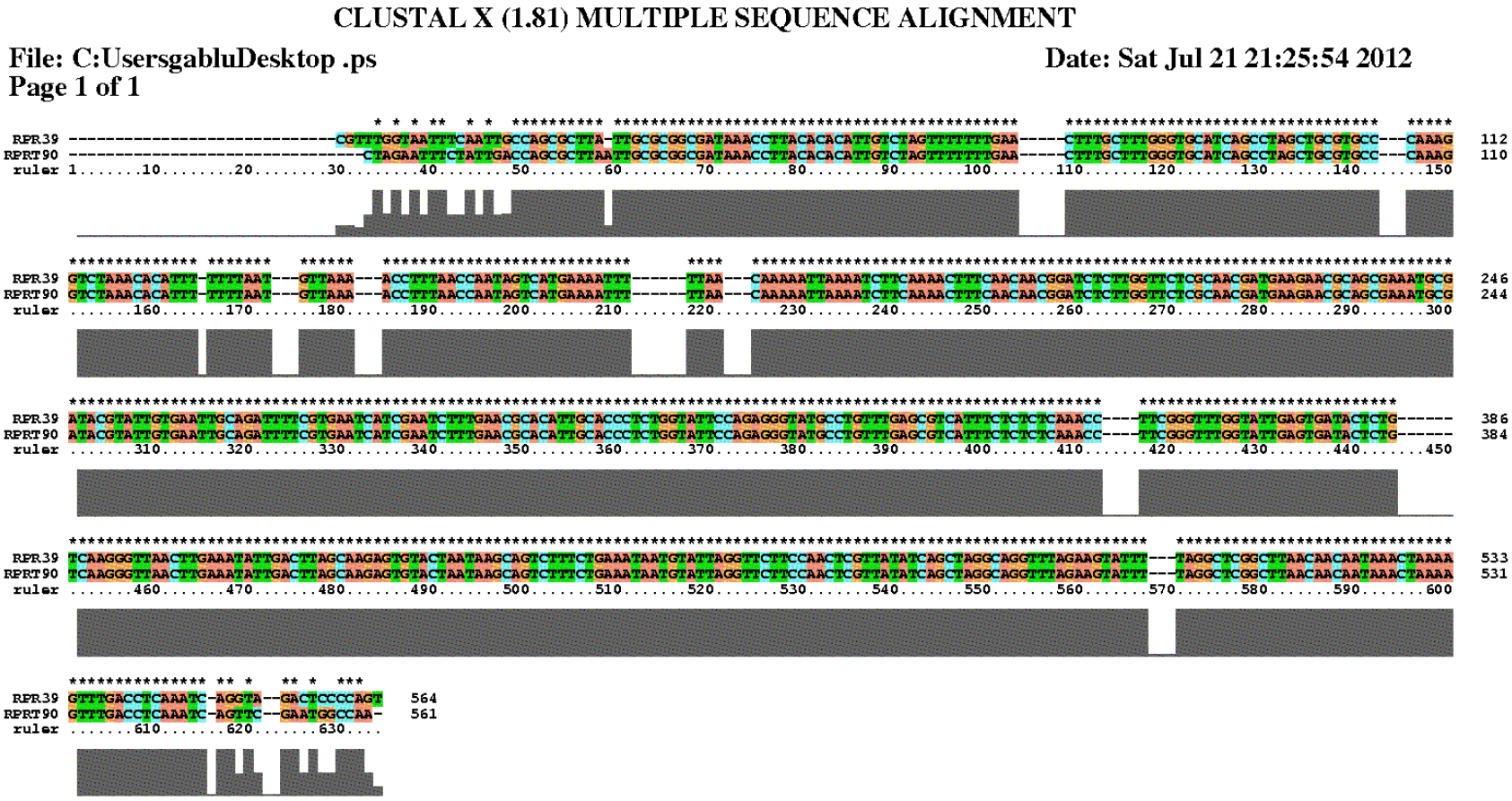


Fig. 28: Multiple sequence alignment data of fusant RPR39 and mutant RPRT90

5.2.7 Ethanol production under multiple stress factors

The study of synergistic effect of stresses is important because a strain may experience multiple stresses simultaneously during fermentation. Besides, it is not possible to clearly separate the effect of one stress condition from another prevailing during fermentation [91]. It has further been reported that the gene expression profile of a yeast strain changes under the effect of stress conditions. Genes involved in various response pathways such as carbohydrate metabolism, detoxification of reactive oxygen species and protein folding get either induced or repressed under various stress conditions and provides ability to yeast cells to adapt or defend themselves in such stresses [128]. This is termed as cross-tolerance i.e. exposure to a mild dose of one type of stress enables yeasts to survive in the lethal dose of same or other stresses. Therefore, the combined effect of stress factors on ethanol production was studied by inducing various stress conditions simultaneously during ethanol fermentation using the most efficient mutant strainRPRT90. The stress tolerance of RPRT90 was also compared with the stress tolerance of strain RPR39. The results of this study are shown in fig 29-34.

From fig 29, it is observed that under the combined effect of temperature (39^oC) and inhibitor (0.25 gL^{-1} vanillin, 0.5 gL^{-1} furfural and 4 gL^{-1} acetic acid), the rate of ethanol production increased with time and the rate was almost constant upto 72 h of fermentation. The strain RPRT90 utilized 88.75 gL^{-1} of glucose-xylose mixture and produced 33.7 gL^{-1} of ethanol (fig. 30). The ethanol yield and productivity were estimated to be 0.379 gg^{-1} and 0.468 $gL^{-1}h^{-1}$ respectively. Under the same conditions, an ethanol production of 23.9 gL^{-1}, ethanol yield of 0.29 gg^{-1} and productivity of 0.331 $gL^{-1}h^{-1}$ were obtained using RPR39. The cell mass increased up to 72 h of fermentation and remained constant thereafter. The maximum biomass of 5.02 gL^{-1} and 3.93 gL^{-1} were achieved using RPRT90 and RPR39 respectively.

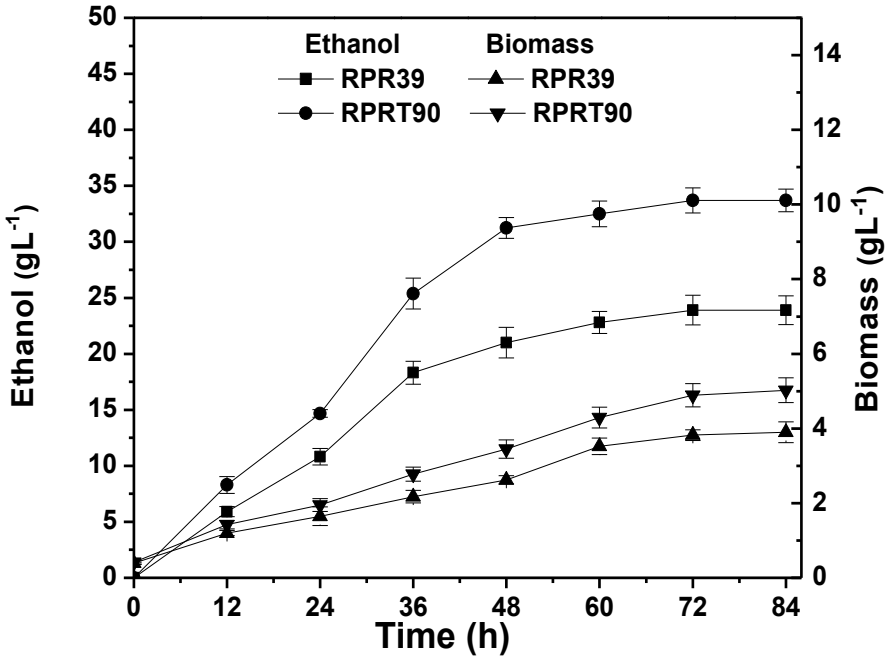


Fig. 29: Ethanol and biomass production under combined effect of thermal and inhibitor stress by mutant RPRT90 and fusant RPR39

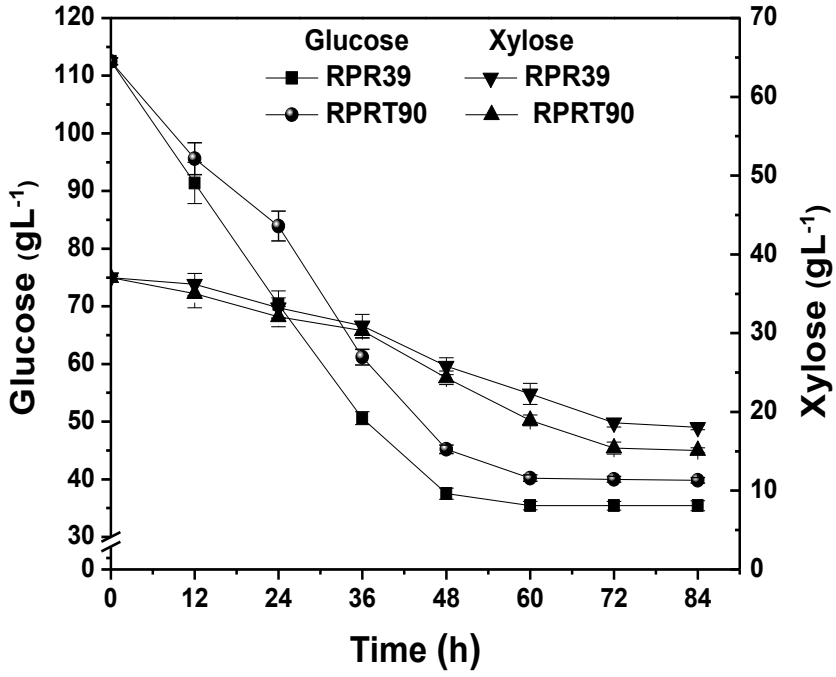


Fig. 0: Glucose and xylose consumption under combined effect of thermal and inhibitor stress by mutant RPRT90 and fusant RPR39

Fig 31 shows the combined effect of ethanol (5%) and inhibitor stress (0.25 gL^{-1} vanillin, 0.5 gL^{-1} furfural and 4 gL^{-1} acetic acid) on ethanol fermentation. The fermentation experiment was conducted at 30^{o}C. Figure 32 indicates that the yeast strain RPRT90 utilized 92.5 gL^{-1} of glucose-xylose mixture out of 150 gL^{-1} and produced 39.8 gL^{-1} of ethanol after 60 h of fermentation. No further increase in ethanol production was observed with the increase in fermentation time beyond 60 h. The ethanol yield and productivity calculated for RPRT90 under stress conditions were 0.431 gg^{-1} and 0.63 $gL^{-1}h^{-1}$ which are much higher than the ethanol yield (0.257 gg^{-1}) and productivity (0.363 $gL^{-1}h^{-1}$) obtained with RPR39. The cell mass was observed to increase up to 72 h of fermentation and then it became almost constant with further increase in fermentation time (fig 32). The maximum biomass of 6.08 gL^{-1} and 4.16 gL^{-1} were achieved using RPRT90 and RPR39 respectively.

The study of combined effect of all three multiple stress factors were also carried out at 39^{o}C with 250 gL^{-1} of glucose-xylose mixture (3:1 ratio) as the carbon source. The medium contained 5% (v/v) of ethanol and 0.25 gL^{-1} vanillin, 0.5 gL^{-1} furfural and 4 gL^{-1} acetic acid. It is observed from experimental results (Fig 33) that the mutant strain RPRT90 utilized 166.76 gL^{-1} glucose-xylose mixture and produced a maximum of 50.03 gL^{-1} of ethanol within a period of 84 h giving 0.3 gg^{-1} ethanol yield, 0.59 $gL^{-1}h^{-1}$ productivity and 68.1% sugar conversion. In comparison, the ethanol production of fusant RPR39 was adversely affected by the various stress factors. In case of RPR39, the ethanol yield, productivity and sugar utilization were obtained as 0.23 gg^{-1}, 0.44 $gL^{-1}h^{-1}$ and 41.2% respectively. The time course profile of fermentation in stressed condition shows that the ethanol production continued till 84 h of fermentation while the cell growth stopped after 60 h for both the strains (Fig 33 and 34). This phenomenon can be attributed by the fact reported earlier that the ethanol concentration that completely inhibits fermentation is higher than that able to inhibit the growth as the glycolytic enzymes are more ethanol tolerant than other enzymes involved in cell growth [129]. In all the above studies on ethanol production from glucose-xylose mixture under stress conditions, the glucose was consumed in the first 20 h of fermentation while xylose consumption started when glucose level reached less than 50 percent. This is in good agreement with the earlier reports on mixed sugar fermentation [130, 5].

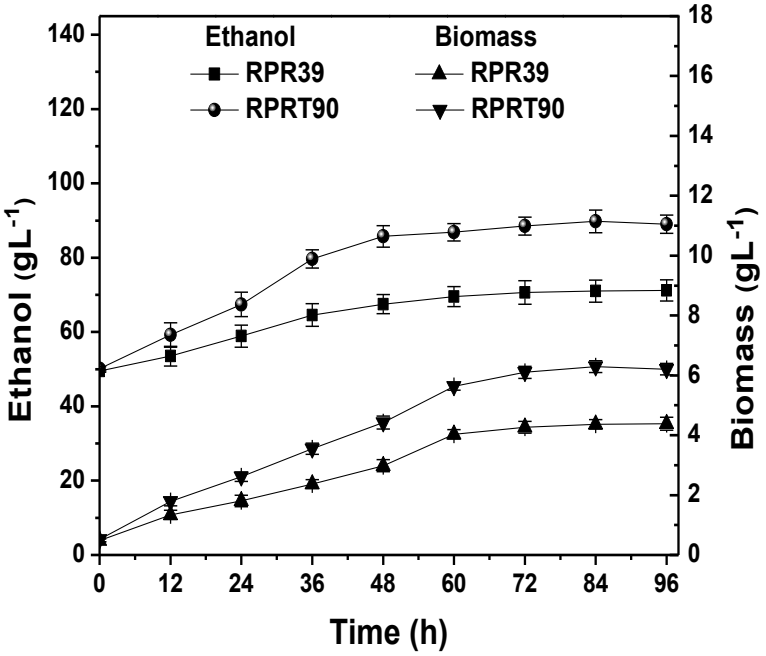


Fig. 31: Ethanol and biomass production under combined effect of ethanol and inhibitor stress by mutant RPRT90 and fusant RPR39

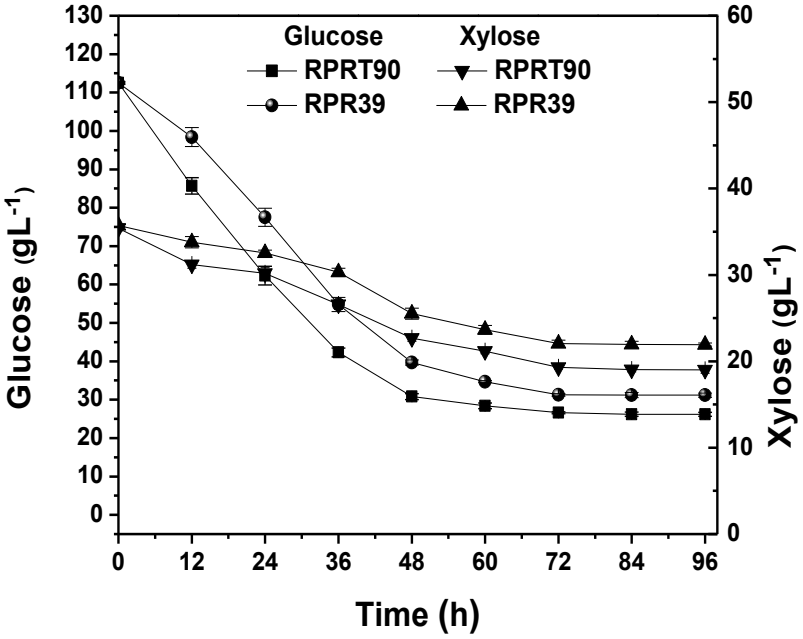


Fig. 32: Glucose and xylose consumption under combined effect of ethanol and inhibitor stress by mutant RPRT90 and fusant RPR39

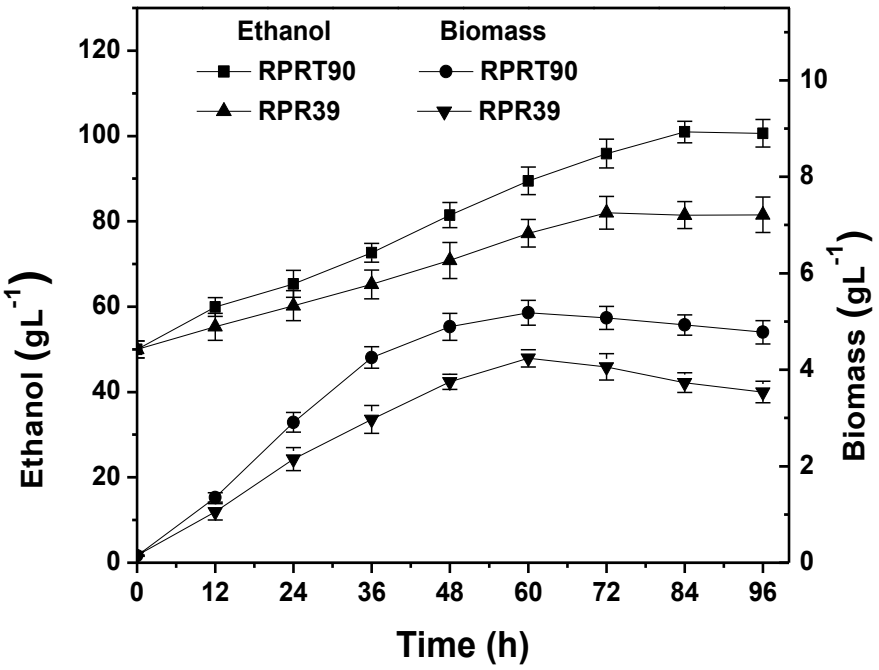


Fig. 33: Ethanol and biomass production under combined effect of ethanol, temperature and inhibitor stress by mutant RPRT90 and fusant RPR39

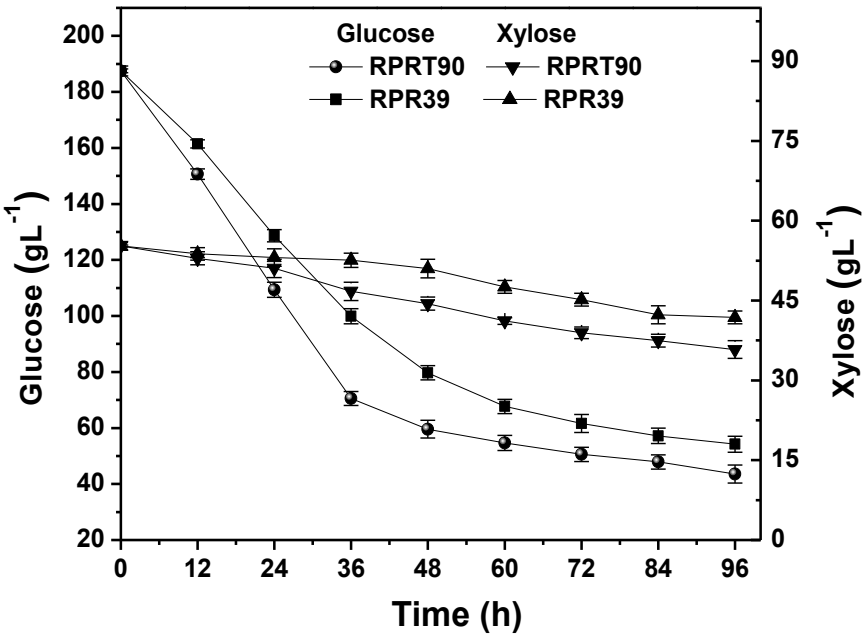


Fig. 34: Glucose and xylose consumption under combined effect of ethanol, temperature and inhibitor stress by mutant RPRT90 and fusant RPR39

5.2.8 Comparison of ethanol production with other reported yeast strains

Several studies have been reported on manipulation of yeast at molecular level to improve their stress tolerance. A comparative study of the published data and the results of present experiment have been shown in Table 11. For example, Sridhar et al. obtained a thermotolerant and osmotolerant *S. cerevisiae* yeast strain UV-VS_3 by UV mutagenesis. The strain was reported to produce 62.0 gL^{-1} ethanol at 40^oC using 250 gL^{-1} glucose as substrate [36]. Sree et al. obtained 64 gL^{-1} ethanol from 250 gL^{-1} of glucose at 40^oC using a thermotolerant strains *S. cerevisiae VS$_3$* [117]. However, the strains VS_3 and UV-VS_3 were not examined for their tolerance to fermentation inhibitors, which is the major obstacle during production of ethanol from lignocellulosic substrates. In another study, Oliva et al. studied the effect of the ternary combinations of acetic acid (0–10 gL^{-1}), furfural (0–2 gL^{-1}) and catechol (0–1 gL^{-1}) on the growth and fermentation of the thermotolerant yeast *K. marxianus* CECT 10875 [84]. The strain produced ethanol with a yield of 0.41gg^{-1} at 42^oC under the ternary effect of 8 gL^{-1} acetic acid, 0.4 gL^{-1} furfural and 0.2 gL^{-1} catechol, whereas in the present study RPRT90 produced ethanol with a yield of 0.398 gg^{-1} in presence of 0.25 gL^{-1} vanillin; 0.5 gL^{-1} furfural; 4 gL^{-1} acetic acid at 39^oC. The high ethanol yield using *K. marxianus* may be due to the fact that the yeast strains belonging to genera *Kluyveromyces* have been reported to be more thermotolerant than *Saccharomyces* strains [131]. Therefore, the growth of *K. marxianus* strain was not much influenced by high temperature.

A recombinant strain R32 developed by genome shuffling techniques was found to produce ethanol with a yield of 0.43 gg^{-1} from 194.4 gL^{-1} glucose under thermal stress (40^oC) and inhibitor stress (0.5% acetic acid) [131]. The same strain produced 78.2 gL^{-1} ethanol at 40^oC in the presence of 0.2% furfural in fermentation medium [132]. However, in the present study, the presence of three different inhibitors resulted in further reduction in ethanol yield. This is in agreement with the earlier report of Lu et al. that the effect of high temperature becomes more deleterious in combination with the inhibitor stress [131]. Moreover, the substrate used in the present study was the mixture of glucose-xylose in place of glucose used in the other studies as reported. The slightly low ethanol yield obtained using RPRT90 may be due to the use of mixed sugar, the fermentation of which is much difficult than the single sugar like glucose. The fermentation of xylose in the fermentation media represses the sugar utilization rate and

thus delays the fermentation [132, 133]. Further, the occurrence of multiple stresses during fermentation also reduced the yield. Many studies have been reported on the fermentation of sugar mixtures, however, to the best of our knowledge; this is the first study on fermentation of mixed sugars under co-stress conditions.

The results indicate that RPRT90 is an efficient strain in terms of both substrate utilization and ethanol fermentation under stress conditions using glucose-xylose mixture as lignocellulosic substrates. The high bioethanol production by RPRT90 even under stressed condition may be due to different mutational events in various metabolic pathways. Thus it is established that multiple induced mutations results in an efficient improvement of the multi-gene regulated characters of yeast such as ethanol production and stress tolerance.

In the present work, multiple mutagenesis has been successfully applied to enhance stress tolerance and ethanol production in a number of mutant strains. The developed mutant showed higher tolerance to initial ethanol concentration and fermentation inhibitors like furfural and acetic acid. Further, the strain showed a comparative growth at 38^{o}C with respect to the fusant RPR39. This is the first report about application of four different mutagenic agents for isolation of mutant strains that were able to tolerate various stresses and showed higher bioethanol production. Thus, the development of this yeast mutant may pave the way for large scale production of bioethanol from various lignocellulosic substrates.

Table 11: Ethanol fermentation by manipulated yeast strains under various stress conditions

Strain	T (h)	t (^oC)	Ei (%, v/v)	Si (gL^{-1})	Sc (gL^{-1})	Inhibitors	Ethanol produced (gL^{-1})	$Y_{E/S}$ (gg^{-1})	Reference
UV-VS_3 100	48	40	0	250	-	-	62	0.24	[36]
VS_3	48	40	0	250	213	-	64	0.3	[117]
K. marxianus CECT 10875	24	42	0	30	-	8 gL^{-1} acetic acid; 0.4 gL^{-1} furfural; 0.2 gL^{-1} catechol	-	0.41	[84]
R32	36	40	0	200	194.4	0.5% acetic acid	84.2	0.43	[132]
R32	48	40	0	200	172.2	0.2% furfural	78.2	0.45	[132]
RPRT90	84	39	5	250	166.76	0.25 gL^{-1} vanillin; 0.5 gL^{-1} furfural; 4 gL^{-1} acetic acid	50.03	0.30	This study
RPRT90	72	30	5	150	92.53	0.25 gL^{-1} vanillin; 0.5 gL^{-1} furfural; 4 gL^{-1} acetic acid	39.8	0.43	This study
RPRT90	72	39	0	150	88.75	0.25 gL^{-1} vanillin; 0.5 gL^{-1} furfural; 4 gL^{-1} acetic acid	33.7	0.39	This study

T, time; t, temperature; E*i*, initial ethanol concentration; S*i*, initial sugar concentration; S*c*, sugar consumed; Y E/S, ethanol yield (gg-1).

PART III-

BIOMASS CONVERSION TO ETHANOL

Ipomoea carnea

5.3.1.1 Introduction

As it is already mentioned that one of the current focus areas in research on biofuel is the production of bioethanol from low cost lignocellulosic biomass. It is also evident from literature that it is difficult to convert lignocellulosic biomass to bioethanol mainly because of the lack of potential microbial strain that can ferment both pentose and hexose sugar components present in biomass [17, 134]. Keeping this in view, a potential hybrid yeast strain was developed in the first phase of dissertation work. In this phase of research work, an attempt has been made to produce bioethanol from weed biomass *Ipomoea carnea* by adopting a fermentation system using the developed hybrid strain. Biomasses widely differ in composition and even the same type of biomass may have different composition due to climatic conditions and seasonal variations. These variations in composition can have a significant impact on their conversion processes to bioethanol.

The result and discussion on the above mentioned research work has been described systematically in this chapter.

Ipomoea carnea, commonly known as bush morning glory is a weed found abundantly in India, Brazil, USA and other countries [135]. This amphibious plant is considered as one of the most productive of macrophytes as it grows profusely on water bodies and adjoining marshy lands. The adaptability of this invasive plant from aquatic to xerophytic habitats made it a highly abundant weed worldwide. The weed causes neurological disorders in livestock on consumption [136], thus it cannot be used as a cattle feed. The wild growth and abundance of this plant makes it a cheaper substrate for production of bioethanol.

Fig. 35: *Ipomoea carnea*

To the best of our knowledge *I. carnea* is an unexploited plant species for the bioethanol production. Therefore, it is essential not only to assess its potential but also to explore the suitable methods for its conversion to produce ethanol. In this study, attempt has been made to investigate the composition of biomass and influence of various methods of pretreatment, enzymatic hydrolysis and fermentation for the exploitation of this new biomass for bioethanol production.

5.3.1.2 Composition analysis

The cellulose, hemicellulose and lignin content of *I. carnea* was determined following the standard methods as described in experimental section and the analytical data are

shown in Table 12. The *I. carnea* biomass was found to contain cellulose, hemicellulose and lignin as the major components. The biomass can be a potential bioethanol feedstock which is evident from its high carbohydrate content of 66%.

The proximate analysis classifies the biomass in terms of its moisture, ash, volatile matter and fixed carbon content based on the complete combustion of biomass to carbon dioxide and water while the ultimate analysis generally includes the estimation of elemental carbon (C), nitrogen (N), hydrogen (H) sulphur (S) and oxygen. The oxygen content (O) is calculated by means of difference. The proximate and ultimate composition of biomass is shown in Table 13.

The biomass elemental analysis is important to evaluate the ratio between the main elements present in biomass, especially C/N ratio. A high C/N ratio implies that the material can be easily burnt and, therefore, suitable for thermo-chemical conversion, on the contrary a low C/N ratio indicates that the biomass is most suitable for biochemical processes [137]. The C/N ratio of *I. carnea* biomass found in the present study is 22.7 which implies that the biomass can be easily converted to bioethanol.

0.18

Table 12: Biomass composition of *I. carnea*

Ethanol-Benzene extractives	**Biomass polymers (wt %)**			**Sugar monomers (%)**			
	Cellulose	Hemicellulose	Lignin	Glucose	Xylose	Arabinose	Other sugars
2.13±0.51	49. 6±2.05	16.4±0.97	24.8±0.74	56.21±0.37	20.3±0.74	14.58±0.84	10.9±0.57

Table 13: Proximate and ultimate composition of *I. carnea* biomass

Proximate analysis (wt %)				**Ultimate analysis (wt %)**				
Moisture	Ash	Volatile matter	Fixed carbon	C	H	N	S	O
4.3±0.36	2.7±0.16	79.24±0.73	12.76±1.34	40.92±1.87	5.27±0.16	1.80±0.62	0.18±0.32	52.33±1.95

5.3.1.3 Acid pretreatment

It is evident from literature that the pre-treatment of lignocellulosic biomass is a pre-requisite step before enzymatic hydrolysis. The role of biomass pretreatment is to remove lignin that binds cellulose and hemicellulose to be hydrolysed and to decrease cellulose crystallinity thereby making cellulose molecules more accessible to hydrolytic enzymes [138]. Several pretreatment methods such as acid [55, 57], alkali [44], steam treatment [45], and alkaline peroxide treatments [65] have been used by researchers worldwide. However, acid pretreatment is an effective and the most commonly used method for pretreatment as it facilitates the yield of monomers of hemicellulosic sugars. Hemicellulose forms covalent linkages with lignin through ferulic acids and with cellulose via pectin and breakage of these bonds by acids releases hemicelluloses [139]. Due to ability of hemicellulose to hydrolyse in acids, the acid pretreatment have been used widely for fractionating the components of lignocellulosic biomass. The acid pretreatment has dual advantage of solubilizing hemicellulose and subsequently converting it into fermentable sugars [55]. Therefore, in the present study the biomass was pretreated using different acids and at varying pretreatment conditions.

Selection of acid pretreatment reagent

In this part of research work, a study was performed to investigate the efficiency of different acids in releasing hemicellulosic sugars from biomass and the results are shown in figure 36 (a). The *I. carnea* biomass was treated with different acids (sulphuric, hydrochloric and phosphoric) at varying concentrations (1-4%, v/v) for 60 min at 120^{o}C. It is indicated from experimental results that the sugar yield increased with increase in acid concentration of both sulphuric and hydrochloric acid up to 3% and then decreased with further increase in concentration. With the use of a weaker acid like phosphoric acid, the xylose yield increased steadily with increase in acid concentration. Among the three different acids used, sulphuric acid was found to be most effective in releasing maximum amount of hemicellulosic sugars followed by hydrochloric acid. The maximum xylose yield was obtained using 3% sulphuric acid (174.88 mgg^{-1}) followed by 4% sulphuric acid (153.42 mgg^{-1}) and 3% hydrochloric acid (138.76 mgg^{-1}).

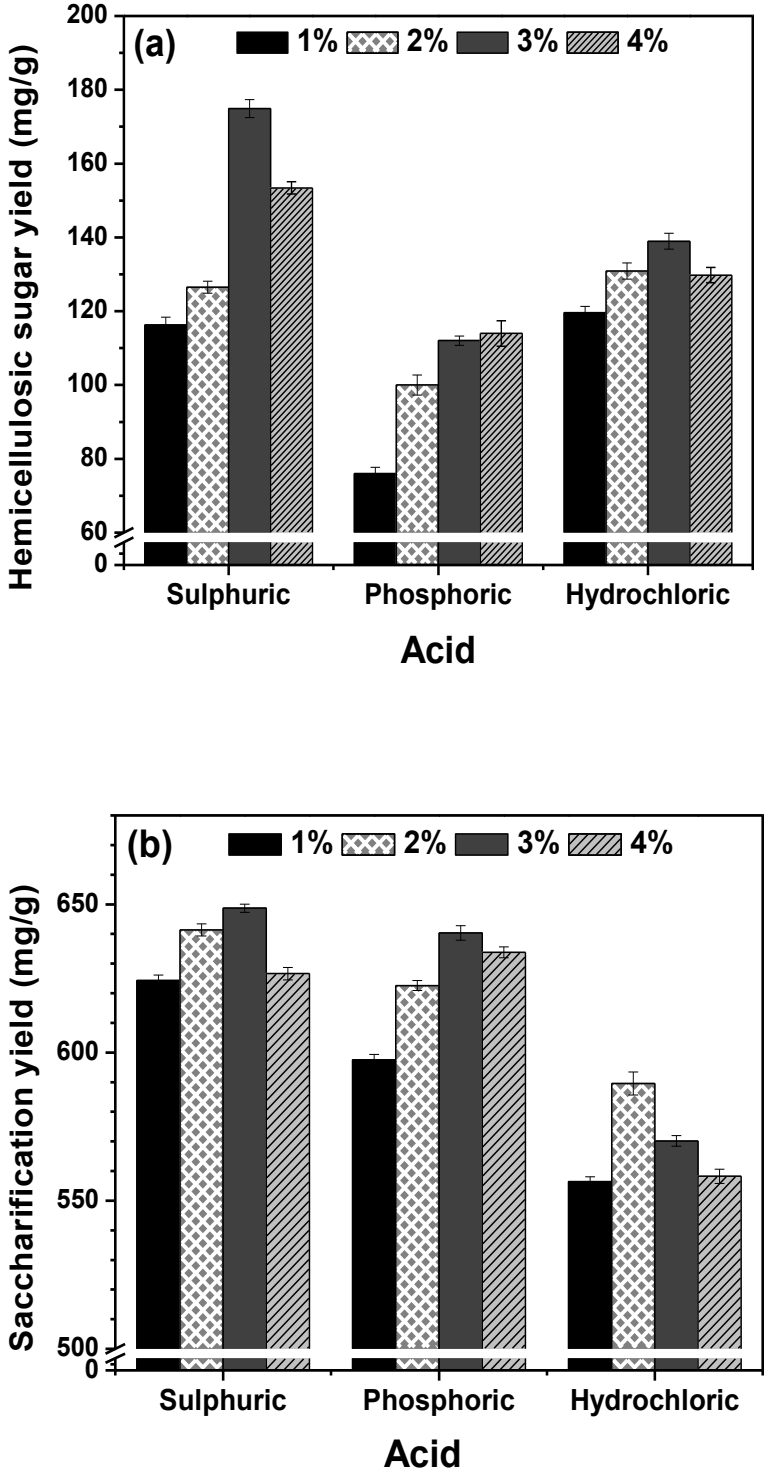


Fig 36. Effect of different acids at varying concentrations on (a) xylose yield from acid hydrolysate and (b) saccharification yield of pretreated biomass

Further, as the severity of pretreatment conditions is reported to have impact on the enzymatic saccharification of the pretreated biomass [140], the saccharification yield of the pretreated biomass achieved using various acid treatments were also compared. The saccharification yield of the pretreated biomass was determined after sulphite delignification and enzymatic hydrolysis. The hydrolysis of biomass for determination of the saccharification yield was carried out using 2.25 FPU/mL cellulase, 7 U/mL β-glucosidase and Tween 20 as surfactant (1%, v/v). The saccharification yield of the pretreated biomass was observed to be severely affected by the use of hydrochloric acid as compared to other two acids used under study. The maximum saccharification yield was found with 3% sulphuric acid (648.69 mgg^{-1} dry substrate) and comparable yield was also obtained with 3% phosphoric acid (640.35 mgg^{-1}). Based on the sugar yield of acid hydrolysate and saccharification yield of biomass pretreated with different acids, it has been established that 3% dilute sulphuric acid is the most effective reagent for the pretreatment of *I. carnea* biomass and thus 3% sulphuric acid was used as pretreatment reagent for further study.

Optimization of dilute sulphuric acid pretreatment

Besides the optimum acid concentration, as described in previous section, the effects of other important pretreatment parameters such as retention time (30-60 min) and temperature (100-140^{o}C) on the constituents of acid hydrolysate were investigated using 3% sulphuric acid to establish the most favourable pretreatment condition. The range of pretreatment time and temperature were chosen on the basis of the results of previous reports on dilute sulphuric acid pretreatment of various lignocellulosic substrates [63, 67, 34, 59]. The acid hydrolysis of the biomass results in the formation of a variety of degradation products like pentose sugars degrade to furfural, hexoses to HMF and lignin degrades to phenolics. These degradation products have been reported to affect the yeast growth and ethanol fermentation by reducing the activities of various enzymes such as alcohol dehydrogenase and pyruvate dehydrogenase. Thus a simultaneous study on the effect of pretreatment severity conditions on the release of fermentation inhibitors was performed. From the experimental results presented in Table 14, it is observed that the xylose content in acid hydrolysate increased with increase in temperature from 100-120^{o}C and incubation time from 30-45 min. The maximum xylose yield of 17.68 gL^{-1} was obtained when the biomass was pretreated at 120^{o}C and 45 min treatment. The

corresponding severity index is calculated as 1.37. Further increase in severity had a detrimental effect on xylose yield. The release of glucose in the acid hydrolysate also showed a similar trend with maximum glucose (3.71 gL^{-1}) release at severity index of 1.5. The total sugar content of the acid hydrolysate, including both xylose and glucose was maximum at CSF 1.5 (21.03 gL^{-1}) followed by CSF 1.37 (20.95 gL^{-1}). At higher severity conditions the glucose content was found to be low due to degradation of glucose to furfural.

The phenolics and furan yield increased with increase in severity and the maximum phenolics (1.052 gL^{-1}) and furans (1.92 gL^{-1}) were generated by treatment at 140^{o}C for 60 min. The saccharification yield of the pretreated substrate was observed to be maximum at 120^{o}C and 60 min (651.78 mgg^{-1}) and a comparable yield was also obtained at 120^{o}C and 45 min (648.21 mgg^{-1}).The saccharification yield also decreased with further increase in severity. Considering both sugar yield of acid hydrolysate and saccharification yield of biomass, the acid pretreatment at 120^{o}C and 60 min using 3% sulphuric acid was found to be most optimum for *I. carnea* biomass.

Table 14: Effect of different pretreatment conditions on the constituents of acid hydrolysate and saccharification yield of pretreated biomass

Temperature (^{0}C)	Time (min)	CSF	Constituent of acid hydrolysate (gL^{-1})				Saccharification yield of pretreated biomass (mgg^{-1})
			Glucose	Xylose	Phenolics	Furan	
100	30	0.63	1.96±0.04	11.64±0.84	0.81±0.13	0.73±0.04	624.43±3.62
	45	0.81	2.35±0.15	13.38±1.01	0.84±0.27	0.89±0.06	635.27±2.58
	60	0.93	2.87±0.09	14.44±0.65	0.98±0.38	1.06±0.17	615.62±3.75
120	30	1.2	3.36±0.12	15.13±0.52	0.94±0.23	1.04±0.05	611.34±3.51
	45	1.37	3.35±0.19	17.68±0.72	1.02±0.17	1.13±0.1	648.21±2.67
	60	1.5	3.71±0.06	17.24±0.84	1.04±0.25	1.25±0.08	651.78±4.25
140	30	1.81	3.58±2.02	16.53±1.02	0.97±0.52	1.47±0.12	638.45±1.38
	45	1.99	3.07±1.83	15.70±0.49	1.05±0.61	1.73±0.11	631.37±3.61
	60	2.11	2.56±1.45	15.45±0.62	1.05±0.52	1.92±0.06	626.74±4.74

Detoxification of acid hydrolysate

It is evident that a variety of toxic by-products are formed during acid pretreatment and these by-products inhibit the yeast growth and fermentation. Therefore, detoxification of acid hydrolysate is essential to avoid the adverse effect caused by various inhibitors. Various methods have been investigated for the removal of fermentation inhibitory compounds like ion exchange [58], overliming [95], ethyl acetate extraction [59], activated charcoal adsorption [61], and laccase oxidation treatment [58]. However, the overliming and the activated charcoal adsorption are the most commonly used methods [61, 63]. Therefore, in the present study, the detoxification of acid hydrolysate was performed using lime and activated charcoal as detoxifying agents both individually and in combinations. Raising of pH using calcium hydroxide results in the transformation of the inhibitory compounds and the use of activated charcoal effectively removes the hydrophobic inhibitory compounds like furan and phenolics. Among these two methods, overliming was found to be more efficient in removing furan and phenolics as compared to activated charcoal treatment. However, the combination of overliming and activated charcoal treatment was found to be highly effective in removing inhibitory compound from *I. carnea* acid hydrolysate. From Table 15, it is observed that the detoxification of acid hydrolysate by overliming was found to reduce the content of inhibitors while further treatment with activated charcoal resulted in almost complete removal of inhibitors. The concentration of phenolics was reduced to 0.054 gL^{-1} from 1.02 gL^{-1}, while furan from 1.13 gL^{-1} to 0.091 gL^{-1}. The corresponding % removal of phenolics and furans were calculated as 94.7% and 91.9% respectively. Furthermore, a small but significant reduction in sugar content (9.1 %) is also observed to be associated with the detoxification process. The loss of sugar of 7.2% and 9.3% during detoxification were also reported earlier in case of *L. camara* and sunflower hull biomass respectively [67,75]. Further, the efficiency of the fermentability of the detoxified and undetoxified hydrolysate was studied and a marginal increase (14.3%) in ethanol concentration was obtained with detoxification (Table 15). In earlier studies with sugarcane bagasse and corn cob, an improvement in ethanol yield of 58% and 90% on overliming and activated charcoal detoxification were earlier reported by Martin et al. and Ge et al. respectively [141, 142]. However, in the present study the improvement in fermentability after detoxification is very less compared to earlier reports. This may

be attributed to the fact that the yeast strain RPRT90 used for fermentation in this study is tolerant to high concentrations of various toxic inhibitors like furfural and acetic acid.

Table 15: Effect of detoxification on sugar content and fermentability of acid hydrolysate

Detoxification treatment	Xylose (gL^{-1})	Phenolics (gL^{-1})	Furan (gL^{-1})	Ethanol (gL^{-1})
None (Undetoxified)	21.03 ± 0.25	1.025 ± 0.17	1.13 ± 0.15	5.28 ± 0.74
Overliming	19.53 ± 0.93	0.392 ± 0.03	0.426 ± 0.08	5.62 ± 0.62
Activated charcoal adsorption	20.14 ± 0.63	0.537 ± 0.06	0.649 ± 0.14	5.43 ± 0.15
Overliming + Activated charcoal adsorption	18.69 ± 0.38	0.054 ± 0.01	0.091 ± 0.06	6.04 ± 0.55

Delignification of pretreated biomass

During acid treatment, though a certain percentage of lignin is removed, most of the lignin remains intact in the cellulosic substrate. The presence of lignin prevents the accessibility of organic components of biomass to the hydrolysing enzyme and hence an appropriate delignification treatment of biomass is essential. Various delignification methods have been exploited in past few decades, alkaline peroxide pretreatment [143], sodium chlorite pretreatment [63], sulphite pretreatment [67] are important among them. In the present study delignification of the acid pretreated biomass was carried out at varying concentrations of sodium sulphite and the percent lignin removal was estimated based on the lignin content of biomass after treatment. As represented in figure 34, a steady increase in the percentage lignin removal was observed with increase in the concentration of sodium sulphite from 10% to 20% (w/v) and sodium sulphite concentration beyond 20% did not show any benefit in lignin removal. Furthermore, the percentage lignin removal was also observed to increase with increase in temperature and incubation time up to 140^{o}C and 45 min. The maximum % lignin removal of 75.6% (251.7 mg/g) was obtained using 20% sodium sulphite at 140^{o}C and 45 min.

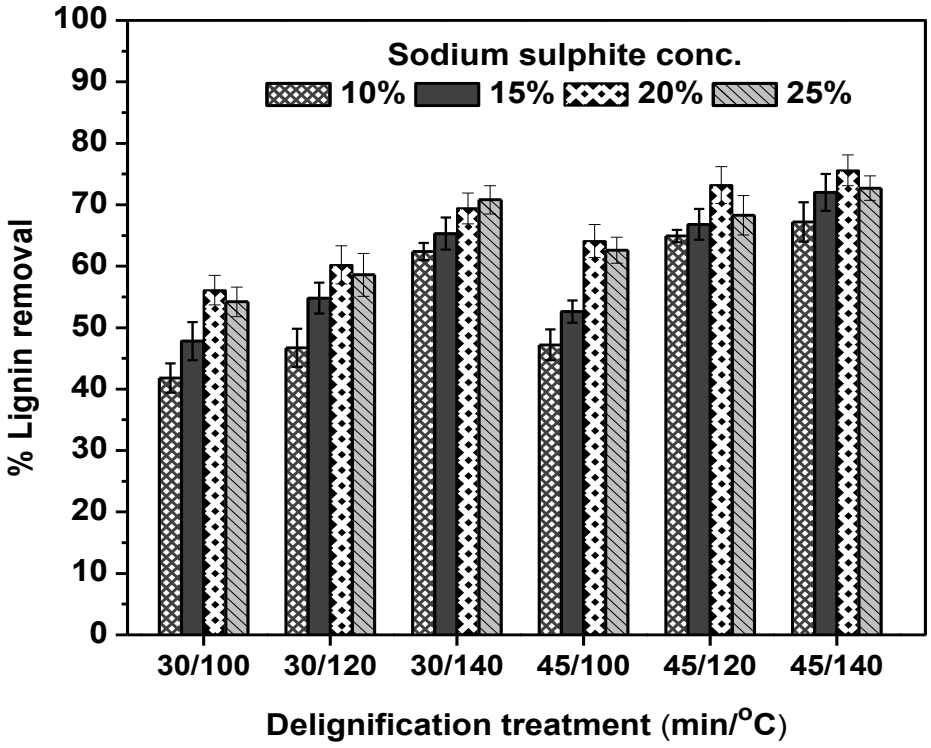


Fig. 37: Percentage lignin removal from biomass under varying temperature, treatment time and concentrations of sodium sulphite

Characterization of native, pretreated and delignified biomass

XRD analysis

The crystallinity index of the biomass in mainly governed by the ratio of crystalline regions to the amorphous regions. The biomass treatments steps like acid pretreatment and delignification remove the hemicellulose and lignin from the biomass. In the present study, the effect of the removal of hemicellulose and lignin on the crystallinity of biomass has been studied by X-ray diffraction analysis (XRD). The XRD spectra of native, pretreated and delignified *I. carnea* biomass are presented in figure 38. It is indicated that the pretreatment of biomass removes the amorphous components while the crystalline cellulose remains unaffected. Thus an increase in the overall crystallinity of the biomass is observed after pretreatment. The characteristic peak of cellulose at 2θ value 22.6^{o} was observed to get sharpened after pretreatment. The sharpening of the peak was associated with the increase in crystallinity index value from 33.75% to

39.5%. It has been reported earlier that the amorphous regions of cellulose are attacked by acids and are responsible for the tranverse cleavage of the cellulose into short chains [139].

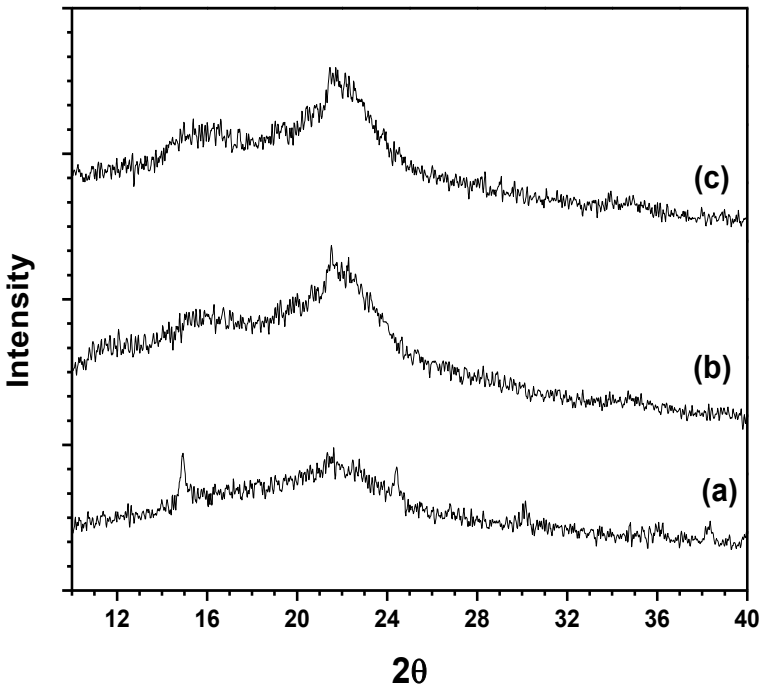


Fig. 38: X-Ray diffraction spectra of (a) native, (b) pretreated and (c) delignified biomass.

Further, the removal of lignin by delignification resulted in more sharpening of peak which corresponds to the increase in CrI value 47.7%. As the amorphous regions of cellulose are interspersed with the crystalline regions, the breaking of amorphous cellulose chains during pretreatment and delignification results in the decrease in degree of polymerization (DP) of cellulose and increase of biomass crystallinity. The breakage of the cellulose polymers to short chains results in exposure of free ends which are liable to enzyme attack which ultimately enhance the enzymatic hydrolysis. The increase in crystallinity index of acid pretreated barley straw and sugarcane tops after pretreatment has also been reported earlier [77, 78].

SEM analysis

SEM study of the native, pretreated and delignified biomass was done to analyze the changes brought in the surface topology of biomass after pretreatment. A study on surface topography is important as both acid pretreatment and delignification process result in the exposure of internal binding sites for action of enzyme during hydrolysis. SEM micrographs of native, pretreated and delignified biomass are shown in fig 39. The micrographs revealed various microstructural variations on the surface of native and treated biomass samples. The SEM image of pretreated sample (Fig 39 b) shows the exposure of the internal structures and formation of multiple pores on the *I. carnea* biomass. The amorphous regions of the cellulose become shredded resulting in the reduction in the size of polymer chains and thus decreasing the degree of polymerization. Acid pretreatment also weakens the van der Waal's interaction between cell wall polymers and removes external fibers [139].

The SEM image of delignified sample (Fig 39 c) shows that the removal of lignin from biomass has made its surface rugged, stripped and defibrillated confirming the disruption of covalent linkages between lignin and cellulose. The loss of intra- and intermolecular hydrogen bonding has resulted in further enhancement in surface area and pore volume. It is believed that the increase the pore volume and surface area of the solid residue due to removal of the hemicellulose and lignin facilitates the access of cellulase to the cellulose structure [144]. The hemicelluloses–lignin matrix that surrounds the cellulose fraction in the biomass has been suggested to act as a physical barrier, which hinders the access of cellulase to the surface of the cellulose. The removal of this barrier during acid pretreatment and delignification increases efficiency of enzymatic hydrolysis [145].

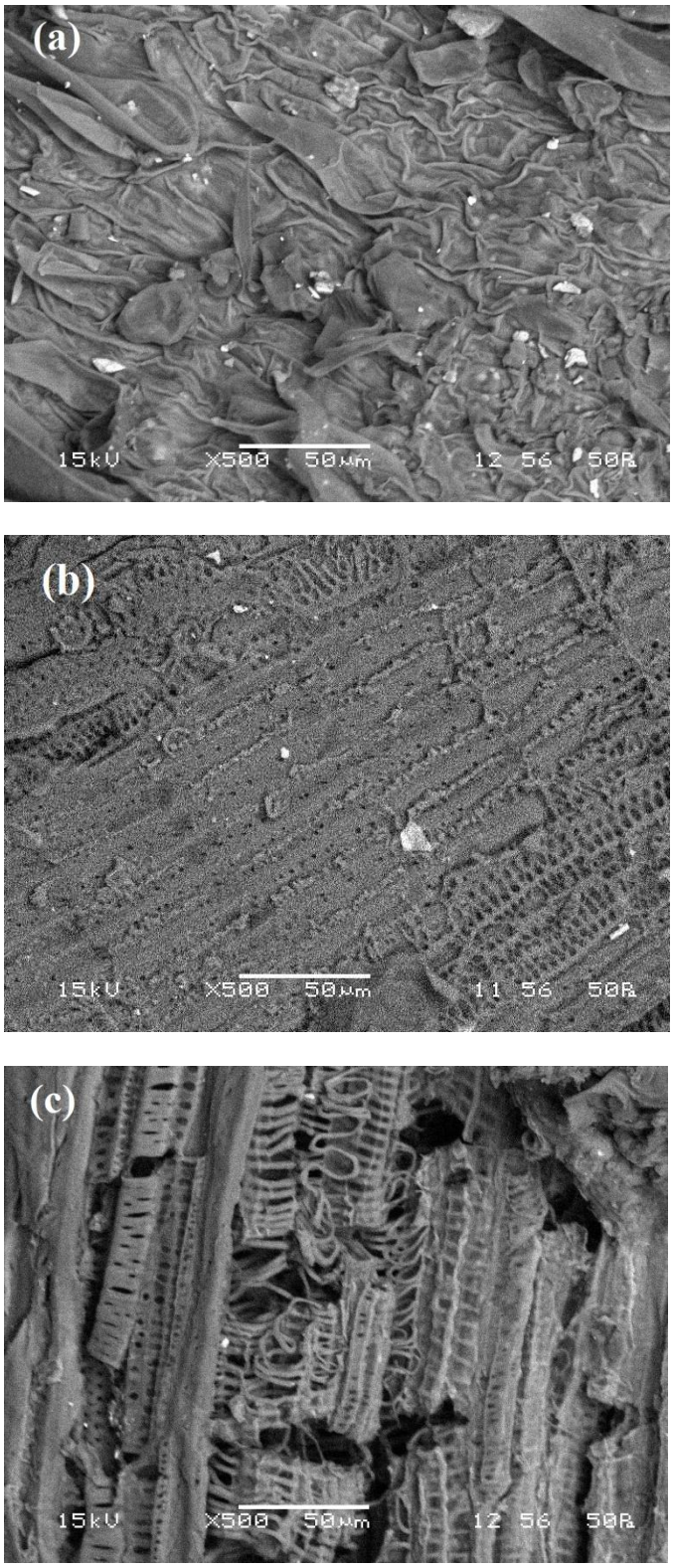


Fig. 39. SEM micrographs of native (a), pretreated (b) and delignified (c) biomass samples of *I. carnea*.

FTIR analysis

The Fourier transform infrared spectroscopy reveals the changes in the functional groups present in biomass before and after the pretreatment. FTIR analysis was performed to investigate the influence of the acid pretreatment and sulphite delignification on the chemical structure of the biomass. The FTIR study is important as it provides the clear indication of the removal of hemicellulose and lignin and the breaking of corresponding bonds by pretreatment and delignification. Fig. 40 shows the FTIR plots of the native, pretreated and delignified biomass of *I. carnea*. The absorption band at 893 cm^{-1} originates from C-O-C stretching at the β-glycosidic linkages between the sugar units in the cellulose and hemicellulose. Absorbance by hydroxyl groups (–OH) in lignin or the C–OH bending in hemicelluloses conferred a prominent band at 1060 cm^{-1} [144] in native sample, while in pretreated and delignified samples the band became broadened with formation of doublet peaks. The bands at 1238 cm^{-1} indicating hemicellulose-lignin linkage [67] and 1425 cm^{-1} representing methoxy group of lignin and hemicellulose [146] were also reduced significantly after dilute acid pre-treatment. The band at 1153 cm^{-1} corresponds to the C-C or C-OH bending in cellulose became more prominent with enrichment of cellulose in pretreated and delignified biomass [78]. As compared to lignin bands of native sample, bands at 1501cm^{-1} (aromatic ring of lignin) and 1732 cm^{-1} (acetyl ester bonds) were significantly enhanced in pretreated samples. This phenomenon may be due to the removal of hemicelluloses during pretreatment and the release and deposition of lignin on the surface [147]. However, these bands again disappeared in delignified sample with the removal of lignin.

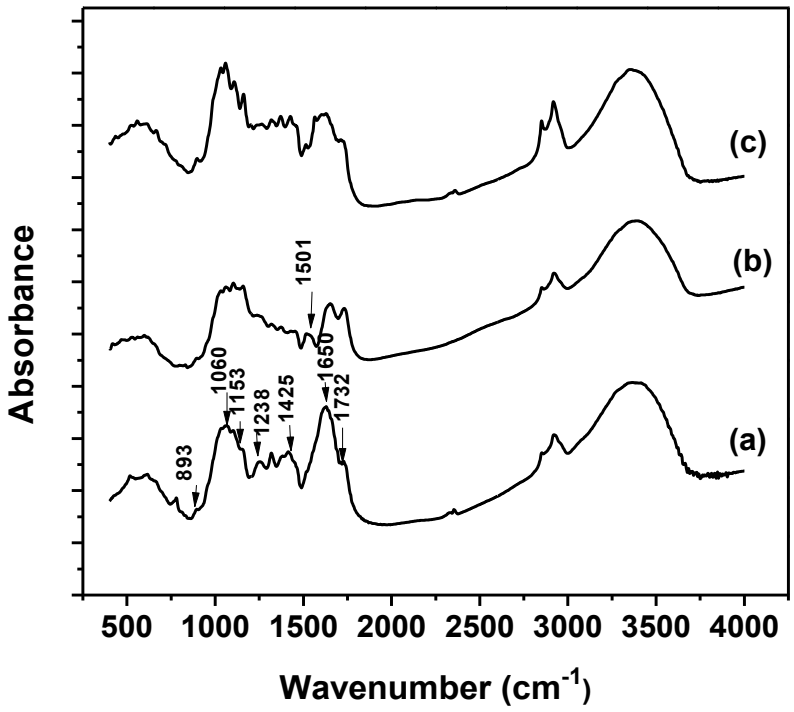


Fig. 40: FT-IR spectra of native (a), pretreated (b) and delignified (c) biomass samples of *I. carnea*.

The absorption peak at around 1650 cm^{-1} became broadened probably due to formation of carbonyl compounds such as ketones and esters. This increase of carbonyl group in lignin changes its chemical structure and makes it more hydrophilic. The broad band at 3350 cm^{-1} was associated with the O–H stretching of the hydrogen bonds of cellulose. The absorption peak in the native sample was similar to that in pretreated solid residues. This implied that most of the crystalline cellulose was not disrupted by the acid-catalyzed reaction. These results were in agreement with a previous study, which reported that the crystalline cellulose in corn stover could not be disrupted by acid hydrolysis [145].

5.3.1.4 Enzymatic hydrolysis of delignified biomass

The cellulase and β-glucosidase (cellobiase) enzymes are reported to hydrolyse the cellulose polymers to free glucose monomeric units. The cellulase catalyse the cleavage of internal bonds of cellulose chain to short chains and β-glucosidase act on the cello-

oligosaccharides and cellobiose to release glucose monomer units from cellobiose [51, 148]. The hydrolysis experiments were conducted to convert the cellulosic polymer of delignified biomass to the monomeric glucose units using cellulase and β-glucosidase enzyme.

The use of surfactant has a great effect on the saccharification process as it inhibits non-productive attachment of exoglucanase to lignin surface and thus allows greater access of saccharifying exoglucanase to cellulose, resulting in high yield of sugar [69, 149]. Surfactants may also have a stabilizing effect on the enzymes, effectively preventing enzyme denaturation during the hydrolysis. Other mechanisms proposed include the surfactant being able to change the nature of the substrate, thereby increasing the available cellulose surface; in turn promoting reaction sites for cellulases [147]. Therefore, the influence of various surfactants like Tween 20, Tween 80 and PEG and their varying concentrations on the release of sugar during hydrolysis were investigated. Among the three surfactants used, the highest saccharification yield (696.2 ±8.3 mgg^{-1}) was obtained with the addition of Tween 20 (T20) as surfactant and a comparable ‘yield (681.3±7.4 mgg^{-1}) was also released using Tween 80 (T80). In this study, PEG was found to be less efficient as compared to Tween 20 and Tween 80.

Further the supplementation of a non-catalytic protein to the surfactants to improve the enzymatic hydrolysis have been suggested by Eriksson et al. Bovine serum albumin (BSA) is known to adsorb to surfaces [149] and has been used to reduce non-specific adsorption in hydrolysis experiments with cellulases. Therefore, a study on the use of chemical surfactants both individually and in combination with BSA has been undertaken and the results are represented in fig 38. BSA was added 1h before adding surfactants and enzymes. It is indicated that a further enhancement in saccharification yield up to 721.7 mgg^{-1} was achieved by the supplementation of BSA with chemical surfactants. The non-hydrolytic protein like BSA binds to the cellulose and results in swelling of microfibrils, thereby decreasing crystallinity and increasing enzyme accessibility. Overall an increase of 28.1 % in sugar yield was observed with the combination of Tween 80 and BSA. Though no significant increase in sugar yield was observed by the addition of BSA to Tween 20 by Erikkson et al. [149], in the present research the addition of BSA before 1h of addition of enzymes and surfactant was found to be effective in enhancing enzymatic saccharification of lignocellulosic biomass.

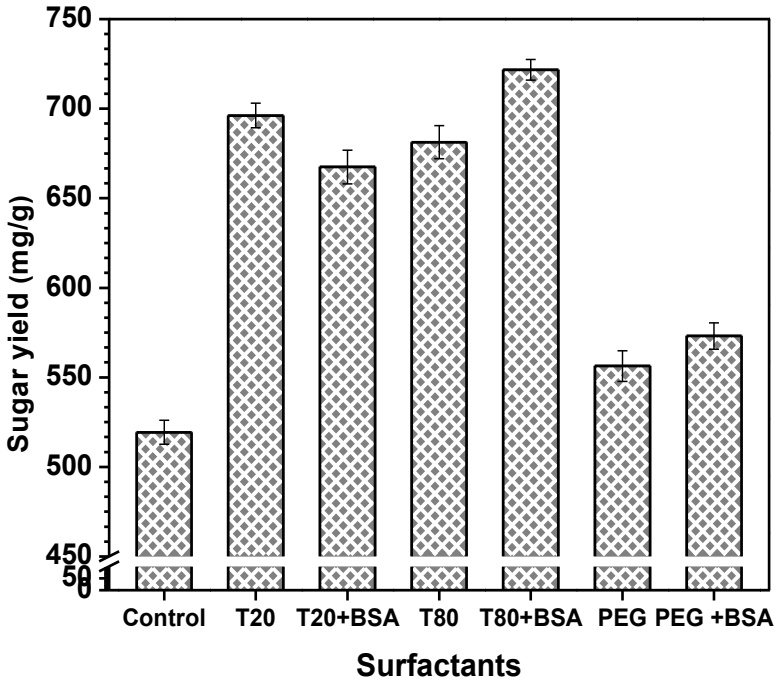


Fig. 41: Effect of types and concentrations of surfactants on enzymatic hydrolysis of *I. carnea* biomass

Optimization of enzymatic saccharification by Response Surface Methodology

The enzymatic saccharification of the pretreated *I. carnea* biomass was optimized by response surface methodology (RSM). The aim of experimental design was to maximize the release of reducing sugars from delignified biomass. The optimization single parameter at a time cannot examine the interaction between the parameters and for the simultaneous optimization of multiple parameters an experimental design tool is essential. The optimization of the hydrolysis experiment by response surface methodology usually involves following steps: i) performing statistically designed experiments, ii) estimating the regression coefficient in a mathematical model, iii) predicting the response and iv) checking the adequacy of the model. A model is an integrated form of various process parameters that is used to describe the entire process. Mathematical modelling of an experiment facilitates the easy manipulation of variables

to be accomplished, with the aim of determining the variations in the process under different situations.

A four-level Box-Behnken Design (BBD) for optimization of four variable parameters has been used. The four variables which are expected to have significant effect on sugar yield and the levels of variables were selected based on earlier reports on enzymatic hydrolysis of other lignocellulosic biomass [150-152]. In the present study, the variables are biomass loading (5-10%, w/v), enzyme loading (1.5-3 FPU/mL), surfactant concentration (0.5-1% Tween 80, v/v) and temperature (45-55^{o}C). Therefore, a three level four factorial design was employed to investigate and validate the process parameters. The response surface curves were plotted to study the interaction of variables and determine the optimum condition of each level which favour the maximum response. MINTAB 16 software was used to analyze the model. Linear and quadratic effects and the possible interactions of the parameters were calculated. Experimental design and experimental sugar yields are presented in Table 16.

Table 16: Yield of sugar for individual runs of the RSM design of enzymatic hydrolysis

Run	Biomass Loading (%, w/v)	Enzyme concentration (FPU)	Surfactant concentration (%)	Temperature (^{o}C)	Sugar Yield (mg/g)
1.	10.0	3.0	0.75	50	662.2
2.	7.5	2.25	0.75	50	729.5
3.	7.5	2.25	0.50	55	654.7
4.	7.5	1.5	0.75	45	643.5
5.	7.5	1.5	1.00	50	644.3
6.	5.0	2.25	1.00	50	577.7
7.	5.0	2.25	0.75	45	571.5
8.	5.0	3.0	0.75	50	648.4
9.	7.5	2.25	0.75	50	740.3
10.	10.0	1.5	0.75	50	588.4
11.	5.0	2.25	0.75	55	621.7
12.	5.0	2.25	0.50	50	558.5
13.	7.5	2.25	1.00	45	638.4
14.	7.5	3.0	1.00	50	687.6
15.	10.0	2.25	1.00	50	572.8
16.	10.0	2.25	0.50	50	622.6
17.	7.5	3.0	0.75	45	681.7
18.	7.5	1.5	0.50	50	672.4
19.	7.5	2.25	0.50	45	631.6
20.	10.0	2.25	0.75	55	633.6
21.	7.5	3.0	0.50	50	669.5
22.	10.0	2.25	0.75	45	612.9
23.	7.5	3.0	0.75	55	678.8
24.	7.5	2.25	1.00	55	668.6
25.	7.5	2.25	0.75	50	733.6
26.	5.0	1.5	0.75	50	574.6
27.	7.5	1.5	0.75	55	681.6

In this study, a second-order model was used to illustrate the relationship between the process variable and response. The polynomial equation for the model used is as below:

$$\textbf{Sugar yield } (gg^{-1}) = 734.467 + 11.675X_1 + 18.617X_2 - 1.658X_3 + 13.283X_4 - 94.854X_1^2 - 21.342X_2^2 - 50.629X_3^2 - 35.642X_4^2 + 0.0X_1X_2 - 17.250X_1X_3 - 7.375X_1X_4 + 11.550X_2X_3 - 10.250X_2X_4 + 1.775X_3X_4$$

where X1, X2, X3 and X4 are the biomass loading, enzyme loading, surfactant concentration and temperature respectively.

Table 17: Analysis of variance (ANOVA) for the response surface model.

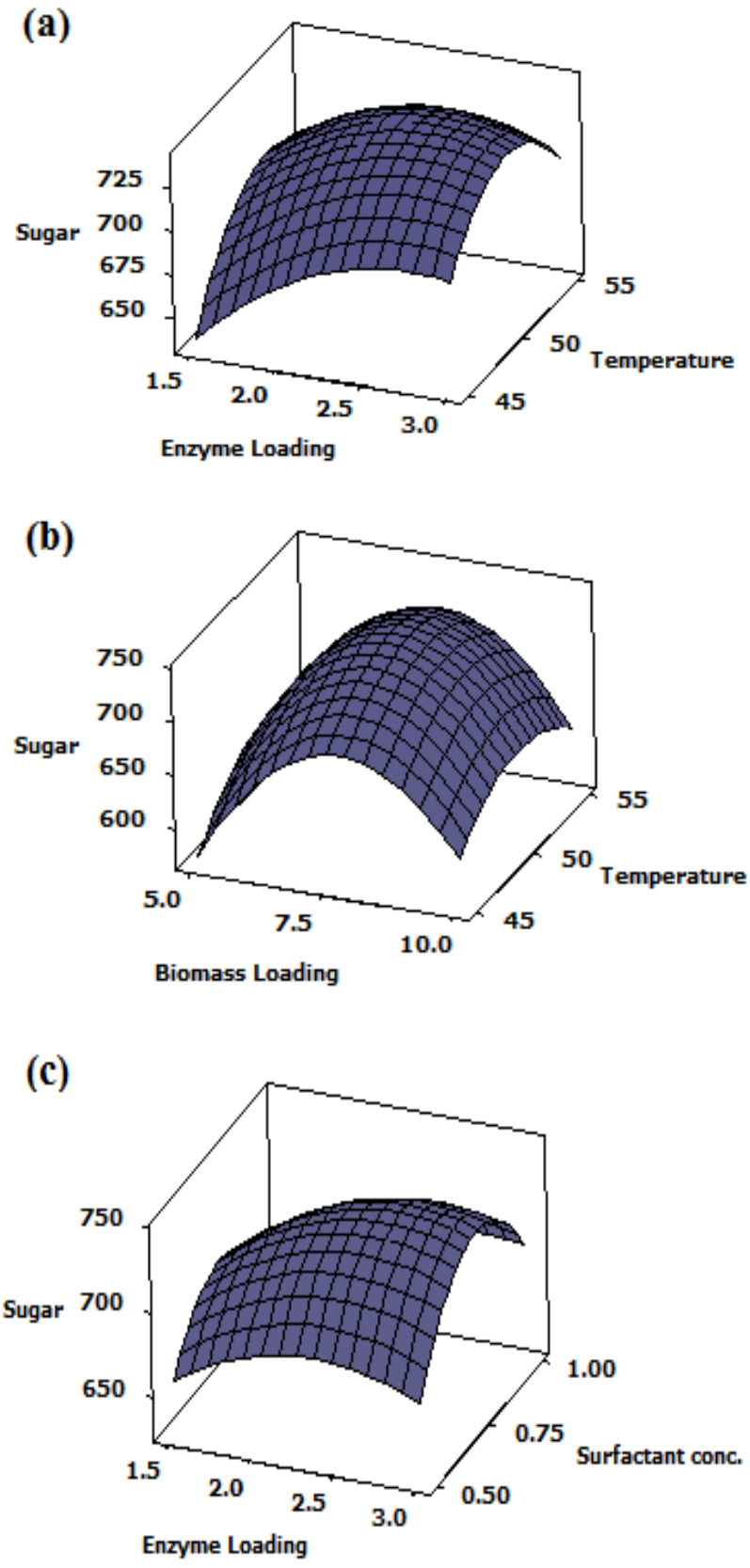
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	61953.6	61953.6	4425.3	16.18	0.000
Linear	4	7945.0	7945.0	1986.2	7.26	0.003
Square	4	51634.4	51634.4	12908.6	47.20	0.000
Interaction	6	2374.3	2374.3	395.7	1.45	0.275
Residual Error	12	3281.7	3281.7	273.5		
Lack-of-Fit	10	3222.3	3222.3	322.2	10.84	0.087
Pure Error	2	59.4	59.4	29.7		
Total	26	65235.4				
R-Sq=94.97%		R-Sq (adj) = 89.10%				

The results of the ANOVA of the polynomial regression equation obtained from the experimental data for the saccharification of delignified samples of *I. carnea* are summarized in Table 17 where the quadratic model depicts the value of coefficient of determination of R square as 94.97%. R square value indicates the variation in the response and in the present study the high R square indicates the robustness of the model and the better fitting of the model with the data. The model adequacy checking includes the test for significance of the regression model. The p-value is usually used as an important parameter to test the significance of each of the coefficient and to understand the interactions between variables. Smaller the p-value, more significant is the correlation with the corresponding coefficient. The ANOVA results shows that the

linear and square terms in polynomial model were highly significant as the p-value was found to be nearly 0. This shows the adequacy of the model to represent the relation between variables and response. The response surface curves were plotted to determine the optimum level of each parameter and their interaction for achieving maximum response. The surface plots showing the interaction between two variables is represented in Fig 42 (a - f).

The response curve representing the interaction between enzyme concentration and temperature is shown in Fig 42 (a). The response curve showed that at lower level of enzyme loading (1.5 FPU/mL), the yield of sugar is low. There is a significant increase in yield of reducing sugar with increase in enzyme loading up to certain level and further increase in enzyme loading reduced the sugar yield. This decrease in yield with high enzyme loading may be due to the feed-back inhibition of produced glucose. The higher concentrations of cellulase results in the accumulation of cellobiose units and the limited concentration of the β-glucosidase limits the hydrolysis of cellobiose oligomers [148]. The middle level of temperature (50^oC) was most optimum for yield of sugars and both high and lower temperatures resulted in a decreased sugar yield. The variations of sugar yield with increasing temperature are in good agreement with Mukhopadhyaya et al. using water hyacinth biomass [152].

Fig 42 (b) shows that at low levels of biomass loading (5%), the yield of reducing sugar is low. Significant improvement in the hydrolysis yield was obtained by increasing biomass loading. When the biomass loading was set at middle level (7.5 %) the sugar yield reached a maximum value and further increase in biomass loading did not show any benefit of increasing sugar level. For enzymatic reaction, fixed substrate concentration is required to reach the adsorption saturation of enzymes and further increase in substrate concentration results in a constant rate of product formation. Biomass loading is considered to be one of the major factors affecting the conversion rate of enzymatic hydrolysis of cellulose [150]. High substrate concentration results in low hydrolysis yield due to product inhibition, enzymatic inactivation, and a decrease in the reactivity of cellulosic substrate with progress of hydrolysis process. A temperature of 50^oC showed maximum response while high temperature resulted in low sugar yield.


(a)
Sugar
725
700
675
650
1.5
2.0
2.5
3.0
Enzyme Loading
45
50
55
Temperature
(b)
Sugar
750
700
650
600
5.0
7.5
10.0
Biomass Loading
45
50
55
Temperature
(c)
Sugar
750
700
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3.0
Enzyme Loading
0.50
0.75
1.00
Surfactant conc.

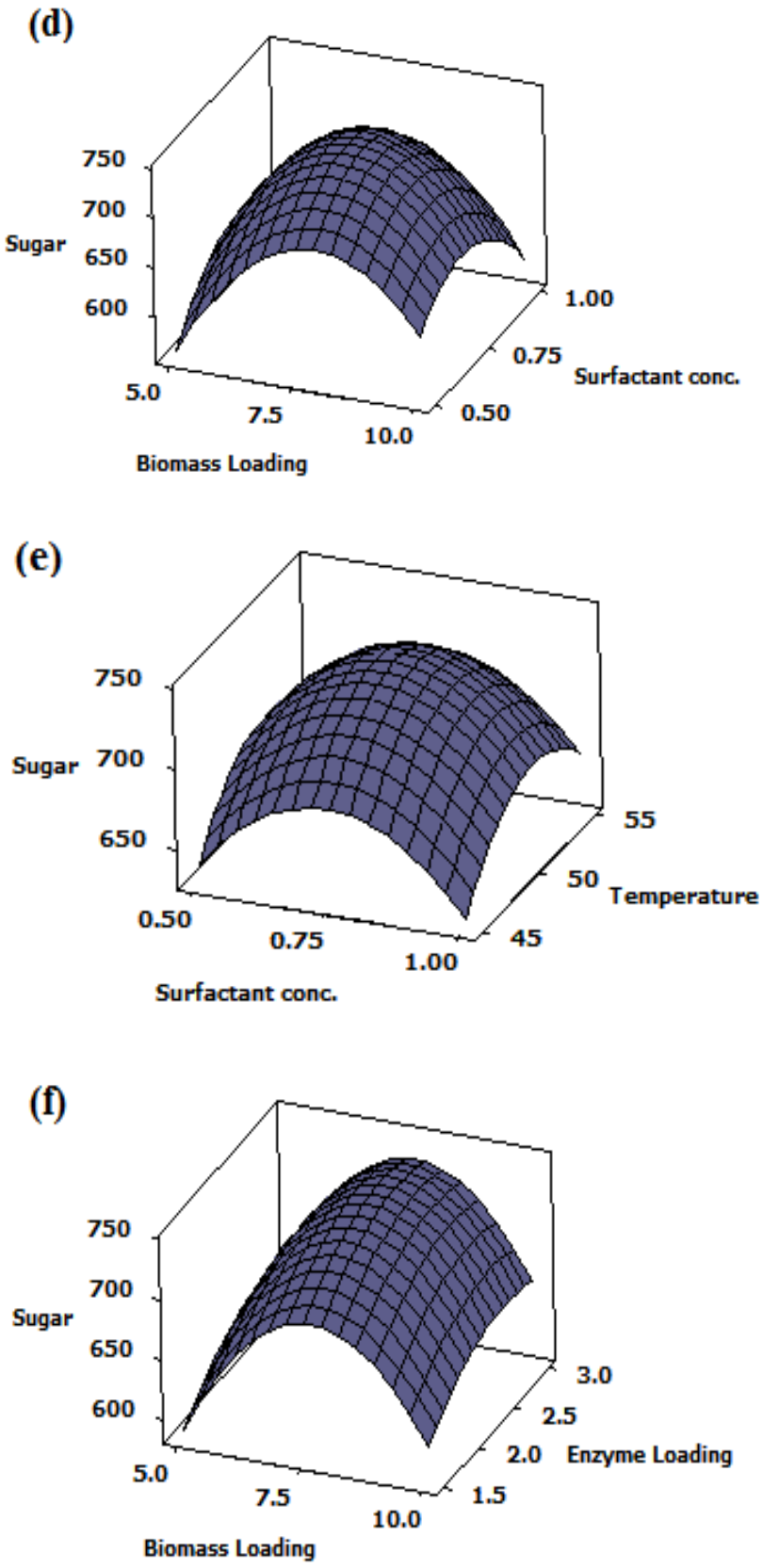


Fig. 42: (a) Response surface plots (a - f) showing effect of interactions various factors on sugar yield of *I. carnea* biomass

Fig. 42 (c) shows that at low enzyme loading, the reducing sugar yield is low. A significant increase in the yield of reducing sugar is observed with an increase in enzyme loading to certain extent. At low level of enzyme loading, increasing the surfactant loading had no effect on reducing sugar yield. This surface plot explains that the middle level of both enzyme loading (2.25 FPU/ mL) and surfactant concentration (0.75%) offer maximum reducing sugars. Similar observations were also earlier reported for enzymatic hydrolysis of wheat straw pretreated with alkaline peroxide [153].

The interaction effects of surfactant concentration and biomass loading on the reducing sugar yield are shown in Fig. 42 (d). Biomass loading is an important factor affecting the enzymatic hydrolysis. It is observed that at low level of biomass loading, increase in surfactant concentration has no effect on the yield of reducing sugars. The reducing sugar yield increased slowly with an increase in substrate concentration from 5% to 7.5%; above 7.5% there was a decrease in the yield of the reducing sugars. This surface plot explains that the middle level of both biomass loading (7.5 %) and surfactant concentration (0.75% Tween 80) shows maximum reducing sugar yield. Different kinds of surfactants have different effects on the stability and activity of enzymes. When the net charge on the enzyme molecule is opposite to that on the surfactant layer, the enzyme molecule interacts with the surfactant layer remarkably and at high surfactant concentration there is a decrease in enzyme activity that may be due to the formation of reverse micelles [150].

Fig 42 (e) explains the interaction between concentration of surfactant and temperature on reducing sugar yield. It is observed that at low level of surfactant concentration the yield of sugars is low. The sugar yield increased with surfactant concentration up to 0.75% and no enhancement in the yield of sugar is achieved on further increase in surfactant concentration. At the middle level of surfactant concentration and temperature the reducing sugar yield is high.

The effect of biomass and enzyme loading on the hydrolysis of *I. carnea* are shown in Fig. 42 (f). At low levels of enzyme and biomass loading, the reducing sugar yield is found to be low and the maximum reducing sugar yield is observed at middle level of biomass and enzyme loading (2.25 FPU). Furthermore, at high biomass loading, the

amount of available free water became less, which in turn decreased the hydrolysis efficiency. High biomass loading is associated with difficulties in mixing as well as end-product inhibition.

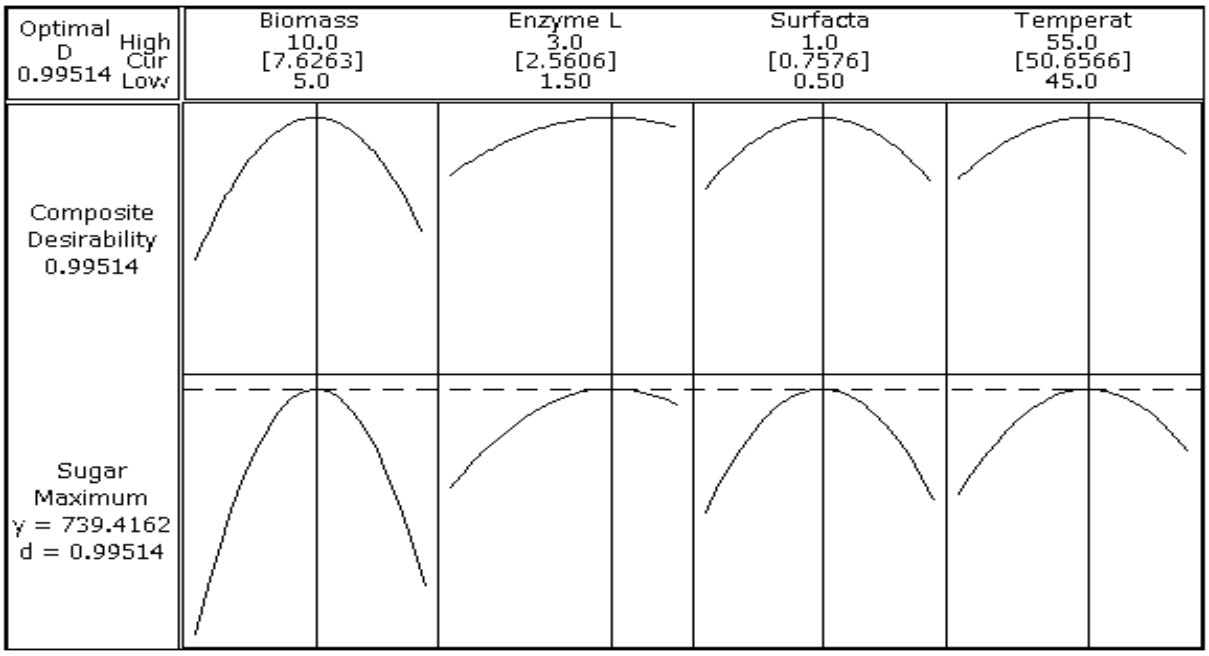


Fig. 43: Optimization plot for sugar yield for enzymatic hydrolysis of I. *carnea* biomass

The predicted optimal values of biomass loading, enzyme concentration, surfactant concentration and temperature were found as 7.6%, 2.5 FPU, 0.75% and 50^{o}C respectively. As indicated in figure 43, the maximum sugar yield at predicted optimum condition was 739.41 mg/g. Confirmation experiment was conducted at predicted conditions and the sugar yield of 746.8 mg/g with sugar concentration of 48.10 gL^{-1} was achieved. The experiment yield is marginally higher than the predicted value. This justifies the accuracy of model and thus the optimization the experiment. The saccharification efficiency was calculated as 78.8%. The percentage saccharification efficiency obtained in the present study is in good agreement with the earlier reports enzymatic saccharification of *R. communis* and rice straw biomass [150, 151].

5.3.1.5 Ethanol fermentation

Fermentation of the mixture of enzymatic hydrolysate and detoxified acid hydrolysate

The ethanol fermentation of the mixed hydrolysate was carried out in a 5L bench top fermenter. The acid and enzymatic hydrolysates of *I. carnea* were mixed in equal amount (2L) and fermentation was carried out using the hybrid strain RPRT90. The fermentation profile is shown in figure 44. It is observed from the experimental results that the fermentation of mixed hydrolysate containing the detoxified acid hydrolysate (18.69 ± 0.54 gL^{-1} sugar) and enzymatically hydrolysed cellulosic hydrolysate (48.10 ± 1.01 gL^{-1} sugars) produced 27.2± 0.96 gL^{-1} ethanol after 28 h incubation. Out of 66.79±0.75 gL^{-1} of sugar, 59.64± 0.69 gL^{-1} was consumed during ethanol production and 7.15±1.03 gL^{-1} was left unutilized which contained 2.16±0.07 gL^{-1} of glucose and 4.99 ± 0.71 gL^{-1} of pentose sugars. The maximum ethanol productivity of 0.971 gL^{-1} h^{-1}, ethanol yield of 0.456 gg^{-1}, % sugar conversion of 89.2% and % theoretical yield of 89.4 % were obtained after 28 h of fermentation. A decline in ethanol production was observed beyond 28 h which may be due to the consumption of accumulated ethanol by the yeast [154]. The yeast biomass yield increased with increase in time and maximum biomass yield of 0.46 gg^{-1} was obtained within 28 h of fermentation. The biomass yield was observed to remain constant thereafter.

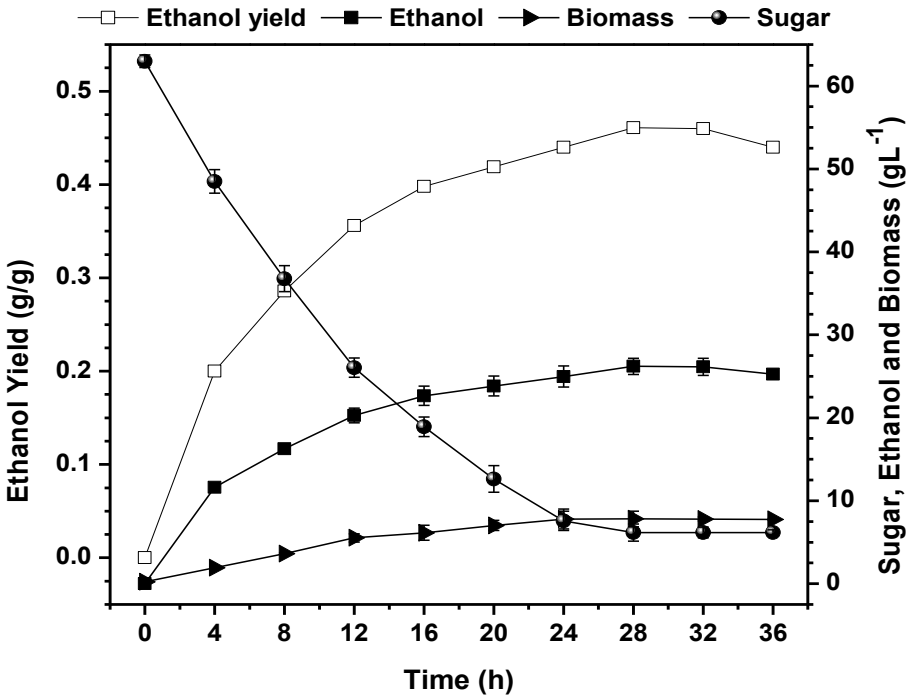


Fig 44: Ethanol fermentation profile of mixture of detoxified acid hydrolysate and enzymatic hydrolysate of *I. carnea* by fusant RPRT90

Fermentation of the mixture of enzymatic hydrolysate and undetoxified acid hydrolysate

The fermentation profile of the undetoxified hydrolysate and enzymatic hydrolysate is shown in figure 45. The sugar content of the mixed hydrolysate was found to be 69.13 ± 0.82 gL^{-1} containing 21.03 ± 0.73 gL^{-1} sugar obtained from undetoxified acid hydrolysate and 48.10 ± 1.01 gL^{-1} sugars from enzymatically hydrolysed cellulosic hydrolysate. The fermentation of total sugar produced 23.05± 0.57 gL^{-1} ethanol after 28 h incubation by utilizing 55.75± 0.28 gL^{-1} of sugar. The % sugar conversion was calculated as 80.6 % while 12.38 ± 0.86 gL^{-1} was left unutilized comprising of 3.81 ± 0.64 gL^{-1} of glucose and 8.57 ± 0.53 gL^{-1} of pentose sugars. The maximum ethanol yield and ethanol productivity achieved after 28 h of fermentation were 0.415 gg^{-1} and 0.821 gL^{-1} h^{-1} and the % theoretical yield was calculated as 81.0 %. Further, increase in fermentation time resulted in a decline in ethanol production. The biomass yield increased with increase in time up to 28 h, reached a maximum biomass yield of 0.447

gg^{-1} and no further increase in biomass was observed with increase in fermentation time (Fig. 45).

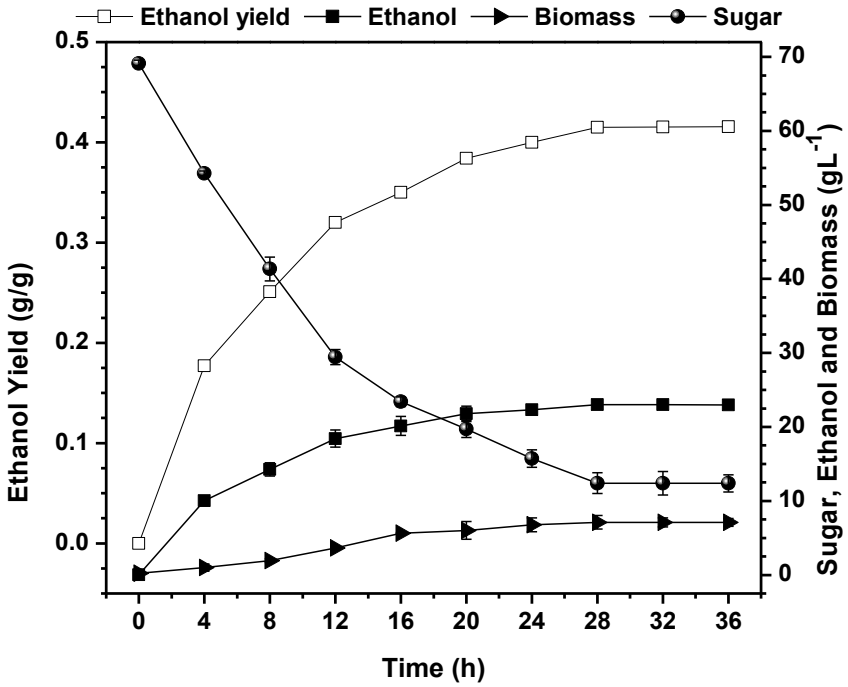


Fig 45: Ethanol fermentation profile of undetoxified acid hydrolysate and enzymatic hydrolysate of *I. carnea* by fusant RPRT90.

The ethanol produced by RPRT90 from mixed hydrolysate containing detoxified acid hydrolysate (27.2 gL^{-1}) was 15.2 % higher than the undetoxified hydrolysate (23.05 gL^{-1}). As mentioned earlier, various studies showed a 58-90% enhancement in fermentability after detoxification. However in the present study the improvement in fermentability with detoxification is very less. This may be because the strain RPRT90 is tolerant to fermentation inhibitors like furfural and acetic acid. Therefore, it is established that the strain RPRT90 can be used to ferment acid hydrolysate even without detoxification.

Lantana camara

5.3.2.1 Introduction

In the previous section, RPRT90 is proved to be an efficient hybrid strain that was successfully employed to produce bioethanol from *I. carnea* weed biomass. In this section, the research work has been extended to assess the efficiency of RPRT90 towards bioethanol production from *L. camar*a which is reported to be a potential lignocellulosic biomass for bioethanol production. The results were compared with the bioethanol production from *I. carnea* as well as earlier reports on *L. camara*. This chapter includes the results and discussion on the above mentioned work.

Lantana camara as lignocellulosic biomass

Lantana camara L. (Verbenaceae) commonly known as red sage is a noxious weed. It is a highly variable species and has been considered as one of the world's top 100 worst invasive species. It can grow at altitudes from sea-level to 2000 m and under a wide range of climatic conditions. It has been cultivated for over 300 years and now has hundred of cultivars and hybrids belonging to the *L. camara* complex [155].

Fig 46. ***Lantana camara***

The diverse and broad geographic distribution of *lantana* is an evidence of its wide ecological tolerances. The approximate total biomass produced by *L. camara* per year

ranges from 15 to 17 tonnes/ha [67]. The abundantly available biomass produced due to its lavish growth makes this weed of potential feedstock for ethanol production.

5.3.2.2 Composition analysis

The composition analysis was done following the standard methods to estimate the cellulose, hemicellulose and lignin content of *Lantana camara* biomass. Further, the proximate and ultimate analyses were also performed for the estimation of moisture, ash, volatile solid, fixed carbon and elemental composition. The results of analytical data are shown in Table 18 and 19.

Table 18: Biomass composition of *L. camara*

Ethanol-Benzene extractives	Biomass polymers (wt %)			Sugar monomers (%)			
	Cellulose	Hemicellulose	Lignin	Glucose	Xylose	Arabinose	Other sugars
2.67±0.20	44.08±1.36	18.12±0.42	28.6±0.82	54.94±0.63	19.14±0.52	14.99±1.58	14.21±0.43

Table 19: Proximate and ultimate composition of biomass

Proximate analysis (wt %)				Ultimate analysis (wt %)				
Moisture	Ash	Volatile matter	Fixed carbon	C	H	N	S	O
3.9±0.41	2.6±0.09	77.38±0.42	16.12±0.18	38.46±2.13	5.04±0.34	2.54±0.17	0.56±0.03	53.4±2.82

The C/N ratio is important for biomass pretreatment, because degradation of lignocellulosic material depends on the material's C/N ratio. The data indicates that with total carbohydrate content of 62.7% and C/N ratio of 15.14, the *L. camara* biomass can be a suitable substrate for bioethanol production.

5.3.2.3 Acid pretreatment

Selection of acid pretreatment reagent

As in the case of *I. carnea*, the *L. camara* biomass was treated with three different acids, sulphuric, hydrochloric and phosphoric acids at varying concentrations of 1-4% (v/v) for 60 min at 120^{o}C to select the most effective pretreatment reagent. Figure 47 represents the efficiency of three acids in releasing hemicellulosic sugars in acid hydrolysate. Among the three different acids used, sulphuric acid was found to release highest amount of hemicellulosic sugars (183.74 mgg^{-1}) followed by hydrochloric acid (154.53 mgg^{-1}).

In the case of sulphuric and phosphoric acid, both hemicellulosic sugar yield and saccharification yield increased with increase in acid concentration up to 4%, whereas using hydrochloric acid, the sugar yield increased up to 2% acid concentration and further resulted in a decline in sugar yield (Fig 47 b). Further, the maximum saccharification yield was observed with the biomass treated with 3% sulphuric acid (634.76 mgg^{-1} dry substrate) followed by 4% sulphuric acid (628.52 mgg^{-1}). As the maximum xylose yield and a high enzymatic saccharification yield was obtained using 4% sulphuric acid, it is established that 4% dilute sulphuric acid is the most effective reagent for the pretreatment of *L. camara* biomass and thus further studies on pretreatment parameters was performed using 4% sulphuric acid.

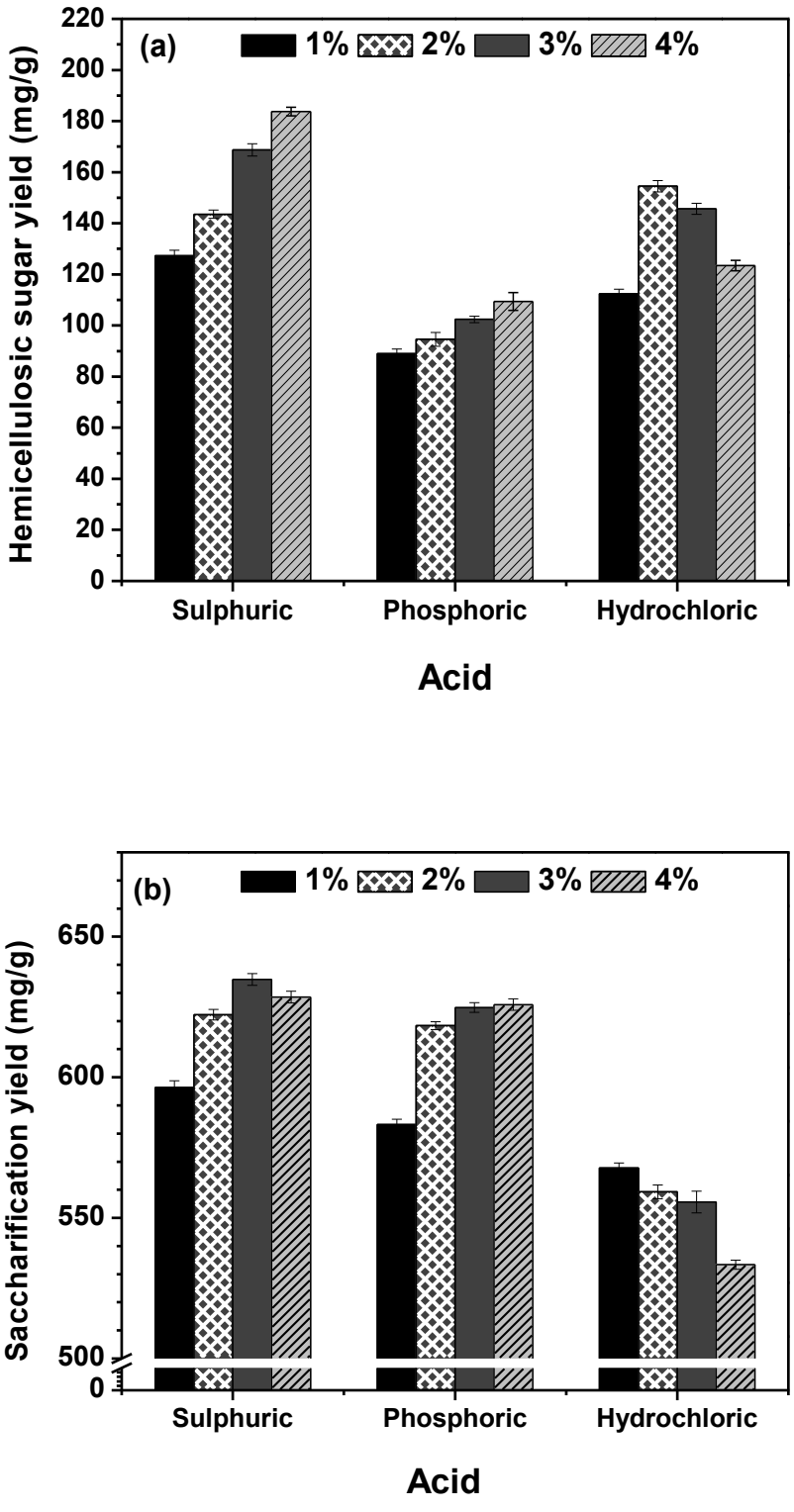


Fig. 47: Effect of different acids at varying concentrations on (a) xylose yield of acid hydrolysate and (b) saccharification yield of pretreated biomass

Optimization of dilute sulphuric acid pretreatment

The dilute sulphuric acid pretreatment of *L. camara* was optimized by studying the effects of various pretreatment parameters such as retention time (30-60 min) and temperature (100-140^{o}C) on the constituents of pretreated solid and acid hydrolysate. From the experimental results presented in Table 20, it is observed that the maximum xylose yield (18.24 gL^{-1}) was obtained when the biomass was pretreated at 140^{o}C for 45 min which corresponds to the CSF of 1.99. A detrimental effect on yields was observed with increase in pretreatment period and temperature beyond optimal conditions of 140^{o}C and 45 min treatment time.

Table 20: Effect of different pretreatment conditions on constituents of acid hydrolysate and saccharification yield of pretreated biomass

Temperature (^{0}C)	Time (min)	CSF	Constituent of acid hydrolysate (gL^{-1})				Saccharification yield of pretreated biomass (mgg^{-1})
			Glucose	Xylose	Phenolics	Furan	
100	30	0.63	0.83±0.14	13.42±0.63	0.52±0.05	0.49±0.06	600.65±4.86
	45	0.81	1.63±0.32	15.56±1.25	0.56±0.07	0.64±0.13	614.48±6.57
	60	0.93	1.72±0.27	15.85±0.36	0.62±0.02	0.86±0.14	620.84±7.83
120	30	1.2	1.52±0.07	16.32±1.15	0.66±0.15	1.00±0.31	611.30±4.73
	45	1.37	1.78±0.20	17.25±1.18	0.68±0.17	1.08±0.37	628.63±8.36
	60	1.5	1.99±0.12	18.14±0.67	0.75±0.09	1.16±0.14	629.59±5.74
140	30	1.81	2.08±0.35	18.06±1.41	0.71±0.13	1.32±0.25	622.63±6.36
	45	1.99	2.00±0.09	18.24±0.72	0.76±0.25	1.32±0.26	630.26±8.86
	60	2.11	1.95±0.16	17.85±1.43	0.84±0.12	1.53±0.35	628.28±9.85

The glucose released in the acid hydrolysate was also found to increase with increase in temperature and maximum glucose release was obtained in the CSF range of 1.81 to 1.99. So, far as toxic inhibitors are concerned, the yield of phenolics and furan has shown to be increased with increase in severity. However, the maximum phenolics (0.84 gL^{-1}) and furan yield (1.53 gL^{-1}) were obtained by treatment with 4% sulphuric acid at 140^{o}C for 60 min. The increase in phenolics release and decrease in sugar yield

at high temperature and prolonged incubation time were also reported by Kuhad et al. in case of pretreatment of *L. camara* biomass [67]. The saccharification yield of the pretreated substrate was maximum (630.35 mgg^{-1}) at 140^{o}C temperature, 45 min treatment time and CSF 1.99 while a nearly similar yield was obtained at CSF 1.5. The yield decreased with increase in severity which may be due to the degradation of sugars to furans (furfural and hydroxyl-methyl furfurals) under severe conditions. Considering all the above factors the acid pretreatment at 140^{o}C and 45 min using 4% sulphuric acid was found to be most favourable for *L. camara* biomass.

Detoxification of acid hydrolysate

The acid hydrolysate of *L. camara* was detoxified using overliming and activated charcoal treatments both individually as well as in combination. Among these two methods when used individually, overliming was found to be more efficient in removing furan and phenolics as compared to activated charcoal treatment.

Table 21: Effect of detoxification steps on sugar content and fermentability of acid hydrolysate

Detoxification treatment	Xylose (gL^{-1})	Phenolics (gL^{-1})	Furan (gL^{-1})	Ethanol (gL^{-1})
None (Undetoxified)	20.96±0.72	0.79±0.61	1.32±0.41	5.70±0.68
Overliming	20.49±0.65	0.163± 0.033	0.45±0.05	5.97±0.53
Activated charcoal adsorption	20.54±0.51	0.344±0.031	0.58±0.08	5.82±0.74
Overliming + Activated charcoal adsorption	19.12±0.35	0.081±0.024	0.21±0.02	6.08±0.73

However, the maximum removal of toxic inhibitors was achieved using the combination of overliming and activated charcoal treatment. From the experimental data shown in Table 21, the concentration of phenolics decreased from 0.79 gL^{-1} to 0.163 gL^{-1} and furan content decreased from 1.32 gL^{-1} to 0.45 gL^{-1} after treatment with lime while further treatment of hydrolysate with activated charcoal reduced the phenolics and furan concentration to 0.081 gL^{-1} and 0.21 gL^{-1} respectively. Overall, the combinatorial effect of overliming and activated charcoal treatment facilitated the removal of furans (84.0 %) and phenolics (89.7%). A small but significant reduction in sugar (8.7 %) was also

associated with the detoxification process. The reduction in sugar after detoxification of lignocellulosic hydrolysate using activated charcoal has also been reported in other studies [61, 63].

A comparison of the fermentability of the detoxified and undetoxified hydrolysate was also made and results shows that the ethanol produced from detoxified hydrolysate (6.08 gL^{-1}) was slightly higher than the undetoxified hydrolysate (5.7 gL^{-1}). The fermentation of detoxified hydrolysate produced 6.08 gL^{-1} which is only 6.25 % higher than the undetoxified hydrolysate. This shows that in the present study the improvement in fermentability after detoxification is marginal. This marginal increase may be because the yeast strain RPT90 is tolerant to various fermentation inhibitors.

Delignification of pretreated biomass

As done in case of *I. carnea*, the acid pretreated biomass was treated with sodium sulphite to remove the residual lignin. The lignin in pretreated biomass reduces the accessibility of hydrolysing enzymes. Therefore, an additional delignification step improves the enzymatic hydrolysis of biomass. From figure 48, it is observed that the % lignin removal increased with the increase in sodium sulphite concentration. The highest removal of lignin was achieved using 20% sodium sulphite and then the % lignin removal from the biomass remained constant even with further increase in sulphite concentration. A study on the variation in lignin removal with various delignification treatments comprising of varying temperature and incubation time revealed that the removal of lignin increased with increase in temperature and incubation time. The maximum % lignin removal of 78.3 % (274.59 mgg^{-1} phenolics yield) was achieved by treating acid treated *L. camara* biomass with 20.0% (w/v) sodium sulphite at 140^{o}C for 45 min. The result is in good agreement with the result of lignin removal of 77% from acid hydrolysate of *L. camara* using sodium sulphite as reported by Kuhad et al. [67].

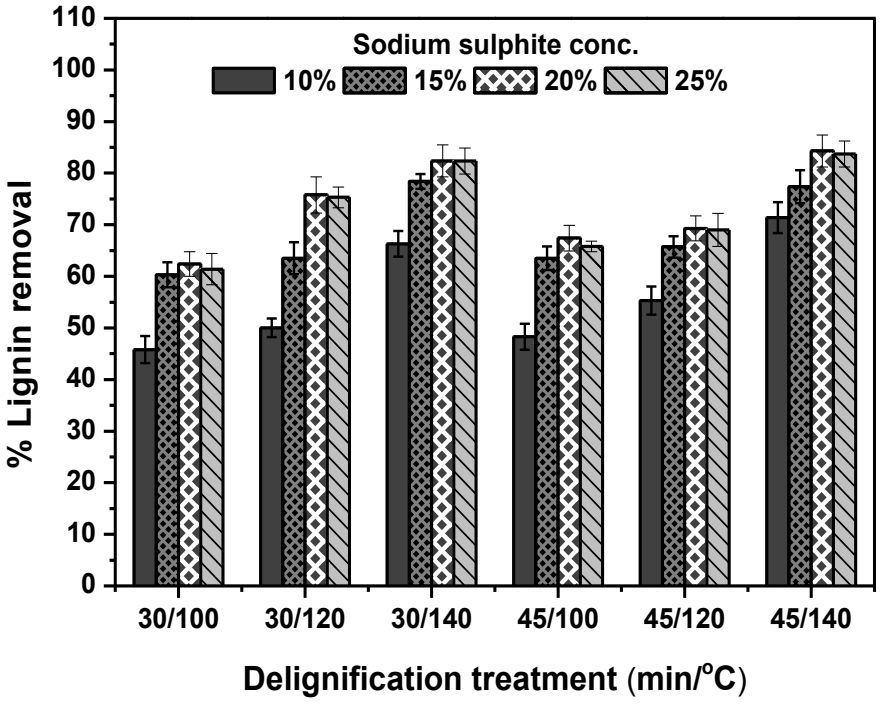


Fig. 48: Percentage lignin removal from biomass under varying temperature, treatment time and concentrations of sodium sulphite

Characterization of native, pretreated and delignified biomass

XRD analysis

The X-ray diffraction analysis (XRD) analyses of native, pretreated and delignified biomass were done to assess the effect of removal of lignin and hemicellulose on the crystallinity of the lignocellulosic biomass. As observed in XRD spectra fig 49, the sharpness in the characteristic peak of crystalline cellulose was found to increase with the removal of amorphous regions of biomass. This indicates that the biomass becomes more enriched with the crystalline units of cellulose. The primary crystalline structure of biomass is obstructed by lignin and hemicelluloses which are amorphous in nature. Thus a significant increase in CrI was observed after the removal of hemicellulose and lignin in delignified sample. The CrI of the native biomass was found as 27.64 % and it increased to 31.53% and 38.42% after acid pretreatment and sulphite delignification respectively. The acid pretreatment also breaks the amorphous cellulose units into short

chains which result in the exposure of free ends. These free ends are then easily attacked by hydrolysing enzymes.

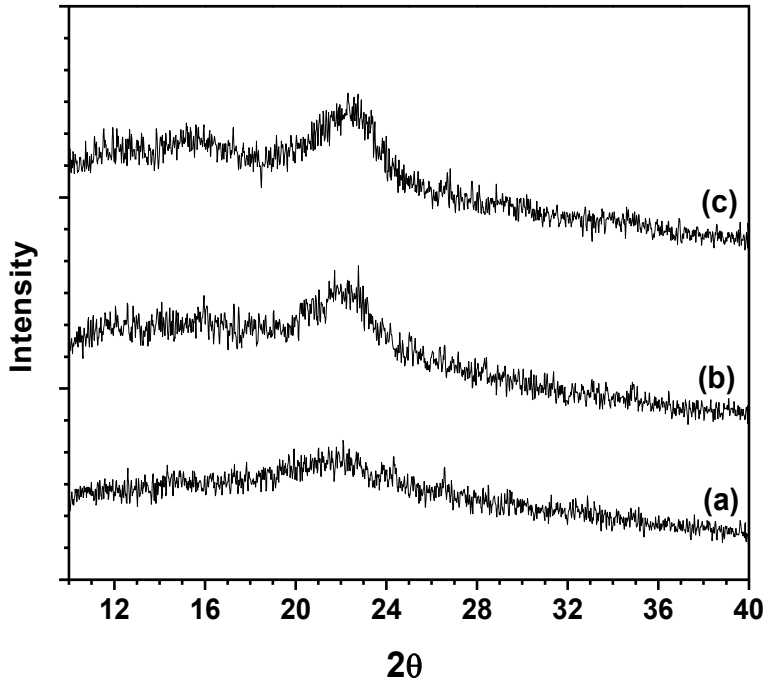


Fig. 49: X-Ray diffraction spectra of (a) untreated, (b) pretreated and (c) delignified biomass

SEM analysis

SEM micrographs of native, pretreated and delignified biomass are shown in fig 45. The SEM image of the pretreated and delignified biomass shows quite variations in the surface topography compared to native sample. The SEM image of pretreated sample (Fig 45 b) shows the formation of multiple pores on the surface. These pores exposure the internal structures and increase the surface area of biomass.

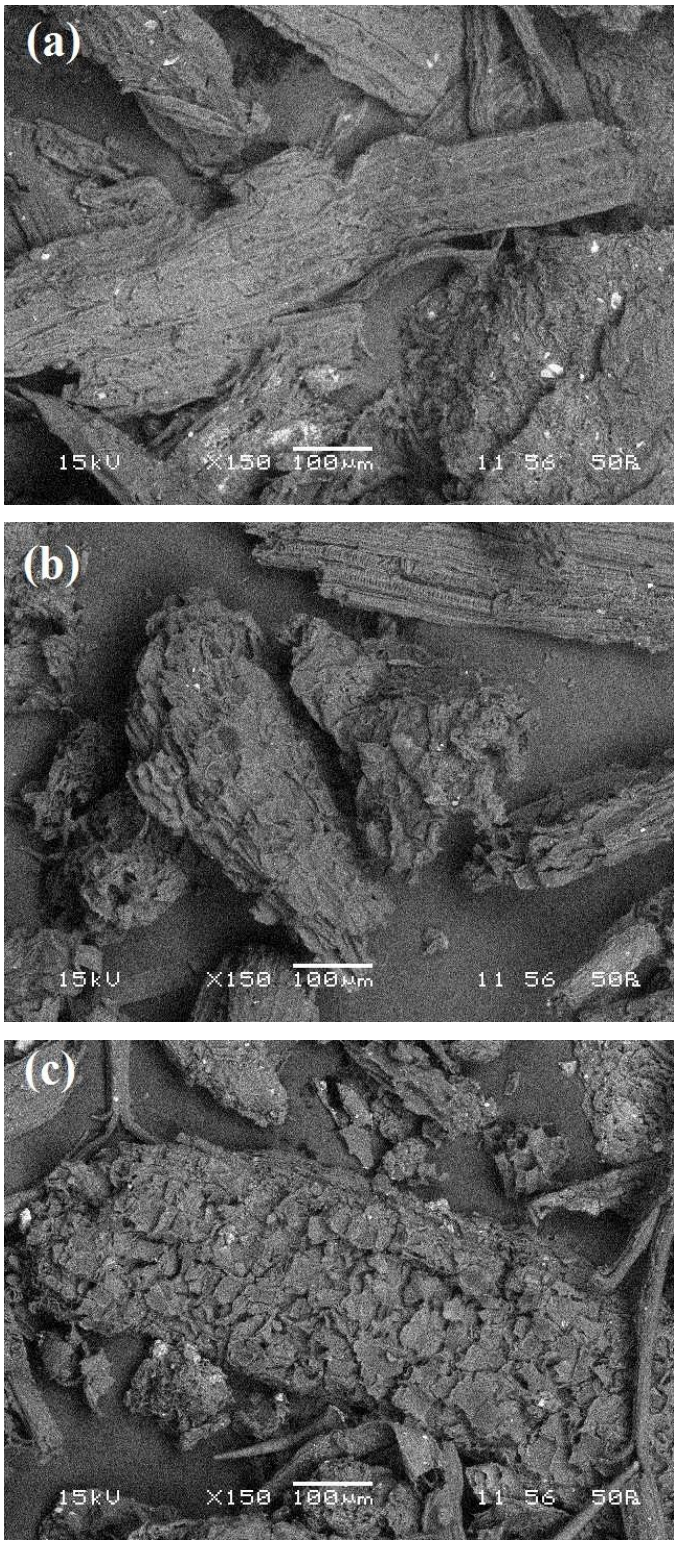


Fig. 50: SEM micrographs of native (a), pretreated (b) and delignified (c) biomass samples of *L. camara*.

The exposure of the internal structures increases the accessibility of hydrolytic enzymes and thus pretreatment of biomass has been found to enhance the enzymatic hydrolysis by increasing the binding sites in cellulose fibers. Furthermore, the surface of the biomass has become defibrillated and stripped after pretreatment. This may be due to the fact that acid dissolve the amorphous zones of cellulose fibres and ultimately resulting in a shortening of fibre length. The breaking of cellulose polymer to multiple oligomeric units results in exposure of more free ends to cellulolytic enzymes. The SEM image of delignified sample (Fig 45 c) shows that the removal of lignin from biomass has made its surface rugged, shredded and defibrillated. The removal of lignin matrix from the biomass results from the breaking of covalent linkages between lignin and cellulose. It is believed that the removal of lignin may further increase the pore volume and surface area of the solid residue. Hence pretreatment and delignification of biomass increase the enzyme accessibility and thus enzymatic hydrolysis of biomass.

FTIR analysis

Fig. 51 shows the FTIR plot of the native, pretreated and delignified biomass of *L. camara*. The band peak at 1060 cm^{-1} originating from the absorbance by hydroxyl groups (–OH) in lignin or the C–OH bending in hemicelluloses is prominently seen in native sample, while in pretreated and delignified samples the band became broadened with formation of doublet peaks. The bands at 1245 cm^{-1} and 1328 cm^{-1} indicating syringyl ring breathing and C-O stretching out of lignin and xylan was reduced in pretreated biomass while in delignified biomass these bands are completely absent. The band at 1440 cm^{-1} representing aromatic C=O stretching from aromatic ring of lignin was divided into multiple peaks in pretreated sample. Further, these peaks were significantly reduced in delignified biomass. The bands at 1501cm^{-1} (aromatic ring of lignin) and 1732 cm^{-1} (acetyl ester bonds) of lignin became prominent in the pretreated samples and then disappeared in delignified sample. This may be because of deposition of lignin on the surface of pretreated biomass and subsequent removal in delignified biomass. The broad band at 3350 cm^{-1} associated with O–H stretching of the hydrogen bonds of cellulose was observed in all the three samples i.e. native, pretreated and delignified biomass.

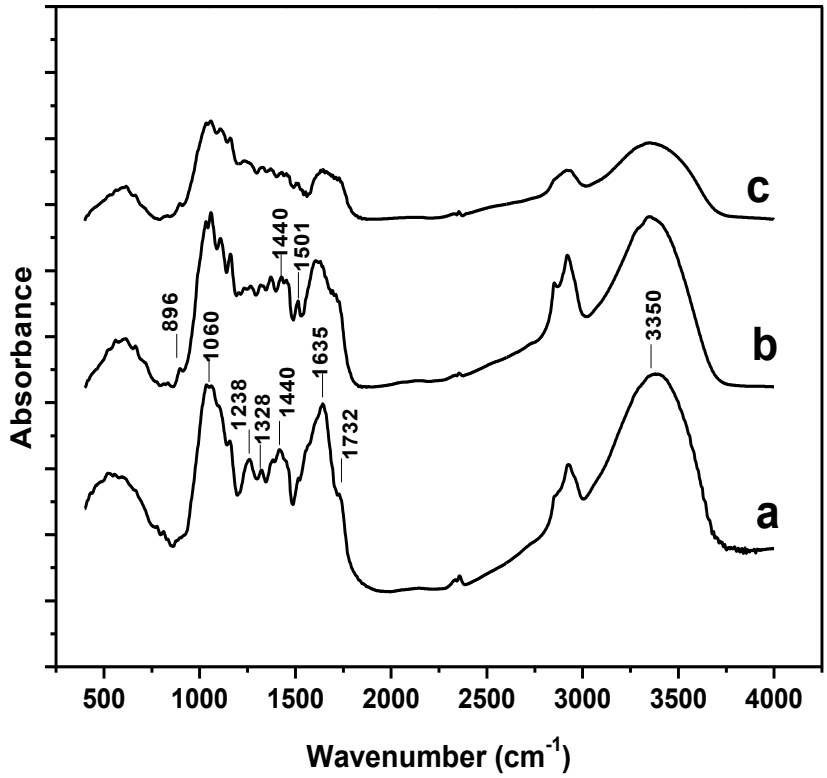


Fig. 51: FT-IR spectra of native (a), pretreated (b) and delignified (c) biomass samples of *L. camara*.

5.3.2.4 Enzymatic hydrolysis

The delignified biomass of *L. camara* was treated with cellulase and β-glucosidase enzyme for the hydrolysis of the cellulose polymers to free glucose monomeric units. Further the effect of a number of surfactant like Tween 20, Tween 80 and PEG has been investigated to enhance the efficiency of enzymatic hydrolysis. A study on the supplementation of surfactants with a non-catalytic protein BSA was also done. The results of enzymatic hydrolysis obtained with and without the use of BSA is represented in fig 52. Among the various surfactants studied, the highest amount of sugar (656.2 ±8.3 mg/g) was released with the addition of Tween 20 (T20) and a comparable amount of sugar (641.3±7.4 mg/g) was also released using Tween 80 (T80). Further, the addition of BSA was found to be very useful as the sugar yield increased to 687.5 mg/g giving an overall increase of 23.4 % with the combination of Tween 20 and BSA.

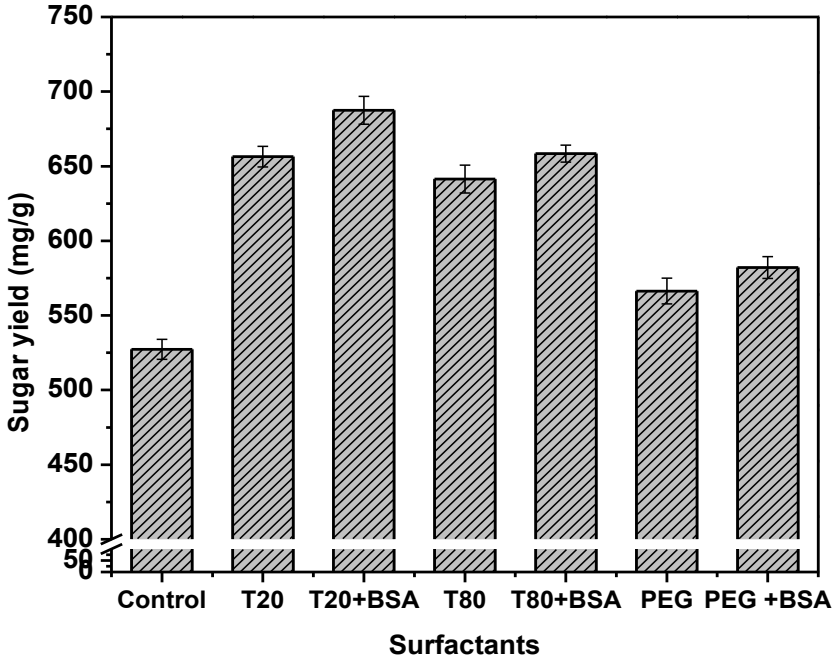


Fig. 52: Effect of types of surfactants on enzymatic hydrolysis of *L. camara* biomass.

Optimization of enzymatic saccharification by response surface methodology

The enzymatic saccharification of the pretreated *L. camara* biomass was optimized by response surface methodology and response surface curves are plotted to study the interaction of key hydrolysis variables and to determine the optimum condition of each level that favours the maximum response. The variable parameters of enzymatic hydrolysis were same as *I. carnea* and the response is the yield of reducing sugars. Experimental design and experimental sugar yields are presented in Table 22.

Table 22: Yield of sugar for individual runs of the RSM design of enzymatic hydrolysis

Run	Biomass Loading (%, w/v)	Enzyme concentration (FPU/mL)	Surfactant concentration (%)	Temperature (^{o}C)	Sugar Yield (mg/g)
1.	10.0	2.25	0.75	50	695.7
2.	10.0	3.0	0.75	50	650.4
3.	12.5	2.25	0.50	55	439.5
4.	10.0	2.25	0.75	45	658.6
5.	10.0	2.25	1.00	50	718.3
6.	10.0	2.25	1.00	50	476.2
7.	12.5	1.5	0.75	45	369.4
8.	10.0	3.0	0.75	50	527.7
9.	10.0	1.5	0.75	50	486.2
10.	10.0	2.25	0.75	50	672.4
11.	7.5	2.25	0.75	55	412.3
12.	7.5	3.0	0.50	50	378.5
13.	7.5	2.25	1.00	45	425.9
14.	7.5	2.25	1.00	50	398.7
15.	10.0	3.0	1.00	50	547.6
16.	12.5	2.25	0.50	50	456.9
17.	10.0	1.5	0.75	45	512.4
18.	12.5	3.0	0.50	50	523.8
19.	7.5	1.5	0.50	45	334.8
20.	10.0	1.5	0.75	55	611.6
21.	10.0	3.0	0.50	50	599.5
22.	12.5	2.25	0.75	45	563.6
23.	10.0	2.25	0.75	55	519.4
24.	10.0	1.5	1.00	55	500.3
25.	12.5	2.25	0.75	50	430.8
26.	7.5	2.25	0.75	50	443.8
27.	10.0	2.25	0.75	55	703.5

The polynomial equation for the model used was as below:

Sugar yield (gg^{-1}) = $705.833 + 32.500X_1 + 34.400X_2 - 5.875X_3 + 60.142X_4 - 203.179X_1^2 - 95.154X_2^2 - 77.767X_3^2 - 40.542X_4^2 + 27.675X_1X_2 + 4.625X_1X_3 + 18.800X_1X_4 - 9.950X_2X_3 - 0.675X_2X_4 - 14.250X_3X_4$

where X1, X2, X3 and X4 are biomass loading, enzyme loading, surfactant concentration and temperature respectively. The polynomial regression equation obtained from the experimental data was used to predict the hydrolysis rate at different levels of the variable parameters within the range of the experimental design.

ANOVA of the polynomial quadratic equation for the saccharification of delignified samples of *L. camara* has been summarized in Table 23. The p-value is usually used as an important parameter to test the significance of each of the coefficient. Smaller the p-value, more significant is the correlation with the corresponding coefficient. The p-value indicates that the linear and quadratic terms in second order polynomial model were highly significant (p> 0.01) and the R square value obtained was 96.44%. This shows the close fitting of the results with the model and its adequacy to represent the relationship between sugar yield and the variable parameters.

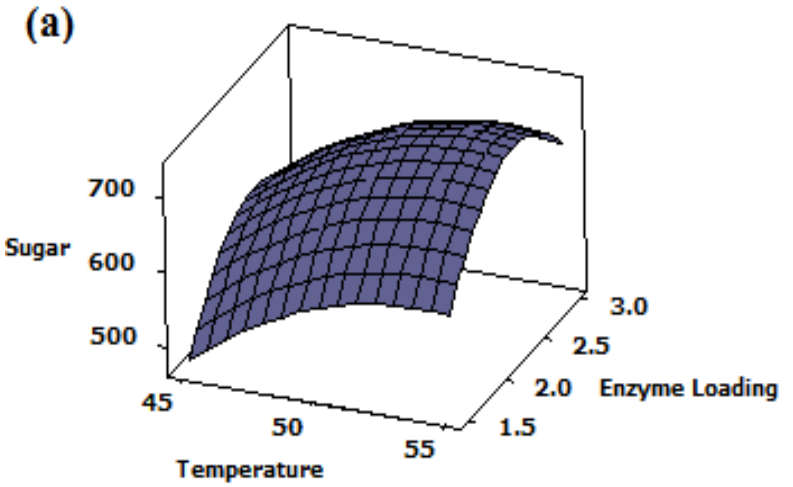
Table 23: Analysis of variance (ANOVA) for the response surface model.

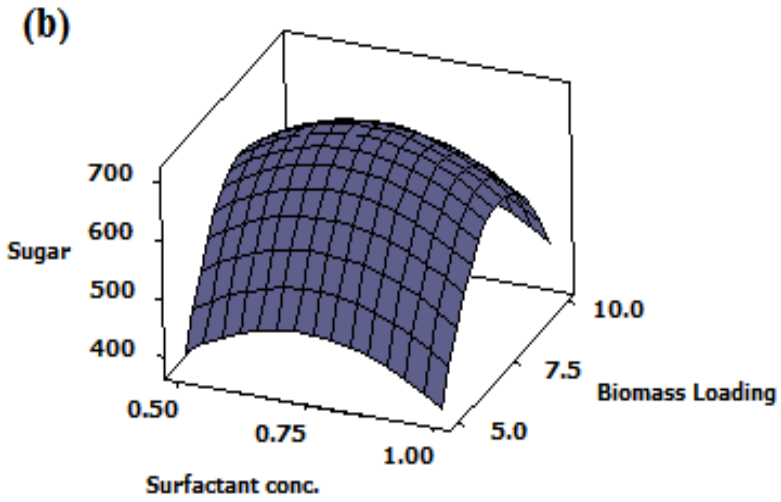
Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	14	309232	309232	22088.0	23.23	0.000
Linear	4	70694	70694	17673.4	18.59	0.000
Square	4	232765	232765	58191.3	61.20	0.000
Interaction	6	5773	5773	962.2	1.01	0.462
Residual Error	12	11410	11410	950.9		
Lack-of-Fit	10	11147	11147	1114.7	8.46	0.110
Pure Error	2	264	264	131.8		
Total	26	320643				
R-Sq=96.44%			R-Sq (adj)=92.29%			

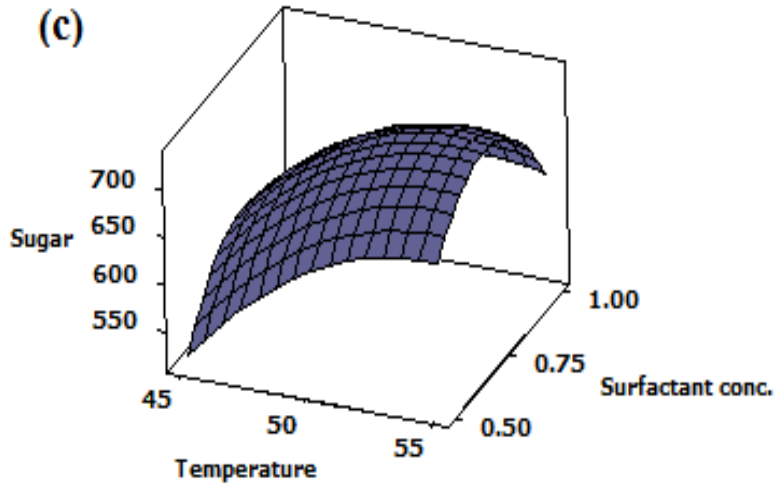
The surface plots showing the interaction between two variables in represented in Fig 53 (a-f).

Fig. 53 (a) represents the interaction among enzyme concentration and temperature. It is evident from the response curve that with the use of lower concentration of enzyme, the yield of sugar was low and it increased with increase in enzyme loading up to 2.25 FPU/mL. Further increase in enzyme loading was not favourable for the enzymatic hydrolysis of *L. camara* biomass. At higher enzyme loading (3 FPU/mL) the yield of sugar decreased which may be because of feed-back inhibition of the product on β-glucosidase and cellulase enzyme. The study of variation in sugar yield with the temperature shows that the sugar yield increased with increase in temperature from 45 to 50^{o}C and no significant change in the sugar yield was observed with further increase in temperature to 55^{o}C. The maximum activity of the cellulase enzyme at 50^{o}C is also reported by many researchers [73, 99].

The interaction of the biomass loading with surfactant concentration (Tween 20) is represented in Fig 53 (b). As indicated from the figure that the sugar yield increase with the increase in biomass loading. The lowest sugar yield was obtained with low biomass loading (5% w/v) while the highest yield was achieved using the middle level of biomass loading (7.5%) and further increase in biomass loading was unfavourable for sugar yield. This may be because of product inhibition and reduction in rate of enzyme-substrate reaction at high substrate concentration. The maximum sugar yield was obtained at 50^{o}C and remained constant after the further increase in temperature.


(a)
Sugar
700
600
500
45
50
55
Temperature
1.5
2.0
2.5
3.0
Enzyme Loading


(b)
Sugar
700
600
500
400
0.50
0.75
1.00
Surfactant conc.
5.0
7.5
10.0
Biomass Loading


(c)
Sugar
700
650
600
550
45
50
55
Temperature
0.50
0.75
1.00
Surfactant conc.

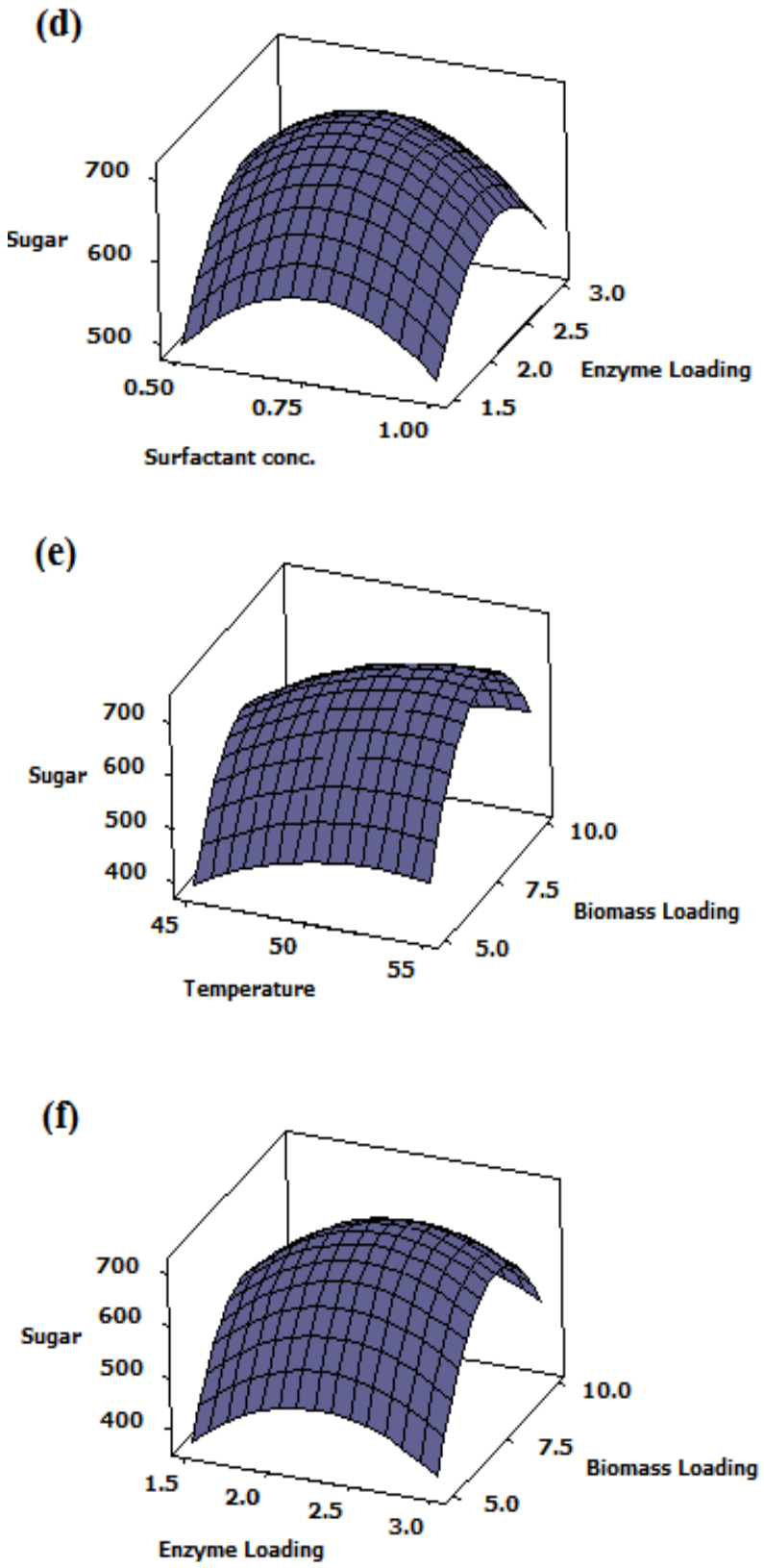


Fig. 53: Response surface plots (a - f) showing effect of interactions of various factors on sugar yield of *L. camara* biomass

The interaction between concentration of surfactant and temperature on reducing sugar yield was also studied and the response surface curve is shown in Fig 53 (c) explains that the low surfactant concentration (0.5% Tween 20) resulted in low yield of sugars. The sugar yield increased with surfactant concentration up to 0.75% and no significant benefit was achieved on further increase of surfactant concentration. The surfactants bind the lignin and thus prevent the binding of enzymes to lignin. The increase in surfactant reduces the non productive binding of enzymes to lignin but at very high concentration of surfactant results in the formation of reverse micelles. The optimum concentration of the surfactant depends on the amount of lignin in biomass and the availability of surfactant binding sites on surface of lignin.

Fig. 53 (d) shows that a similar trend in sugar yield was observed with increasing surfactant and enzyme concentration. The sugar yield increased with the increase in both surfactant concentration and enzyme loading. The maximum sugar yield was obtained at middle level of both variable parameters and the high level of these parameters was unfavourable for enzymatic hydrolysis. At low enzyme loading, the reducing sugar yield was low because of a high substrate to enzyme ratio and the use of high enzyme loading results in feedback inhibition.

The interaction effects of temperature and biomass loading on the reducing sugar yield are shown in Fig. 53 (e). The effect of biomass loading on sugar yield was also similar in the interaction between enzyme loading. The interaction between enzyme and biomass loading is shown in fig 53 (f). Middle level of biomass loading and middle level of enzyme loading (2.25 FPU) showed maximum reducing sugar yield. Higher concentrations of biomass loading was associated with inefficient mixing of enzyme with biomass that ultimately resulted in low sugar yield.

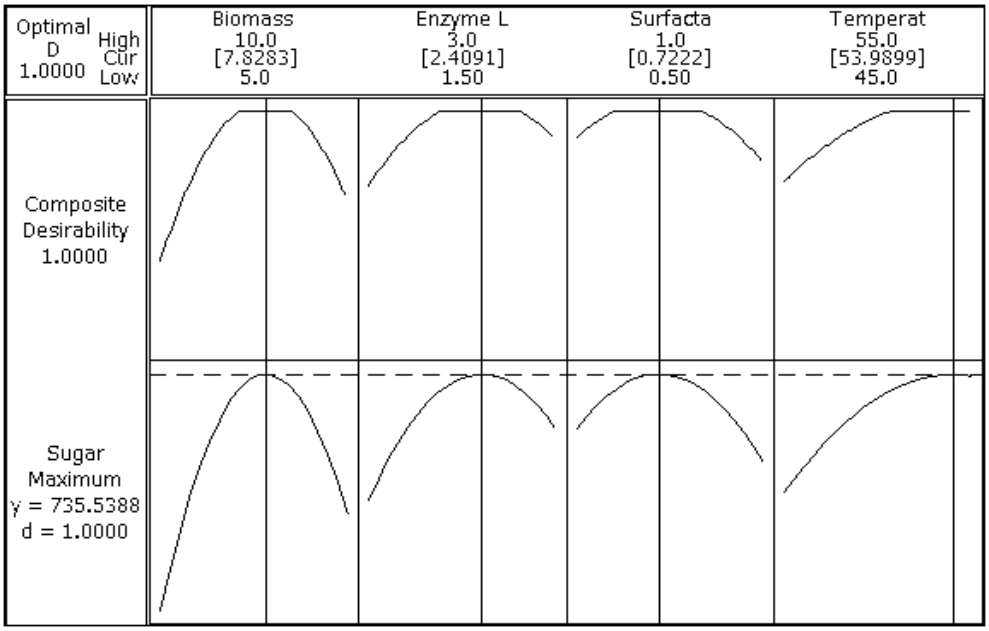


Fig. 54: Optimization plot for sugar yield for enzymatic hydrolysis of *L. camara* biomass

The response optimizer was used to predict the combination of input variables settings that gives maximum response and the optimization plot is shown in figure 54. The predicted optimal values of biomass loading, enzyme concentration, surfactant concentration and temperature were found as 7.8%, 2.4 FPU, 0.72% and 53^{o}C respectively and the maximum sugar yield at this condition was predicted as 735.53 mg/g. A confirmation experiment performed under these conditions provides the maximum sugar yield of 727.3 mg/g. The small difference between model predicted values and experimental value of response justifies the accuracy of model. The saccharification yield was calculated as 75.6%.

5.3.2.5 Ethanol fermentation

Fermentation of mixture of detoxified acid hydrolysate and enzymatic hydrolysate

The detoxified acid hydrolysate and enzymatic hydrolysates of *L. camara* were mixed in equal amount (2L) and fermentation was carried out using the strain RPRT90 and the experimental results are shown in Fig 55. Fermentation of mixed hydrolysate (58.61±0.75 gL^{-1} of sugar) containing the detoxified acid hydrolysate (19.12± 0.38 gL^{-1} sugar) and enzymatically hydrolysed cellulosic hydrolysate (39.49±1.27 gL^{-1}sugars) produced 25.45 ± 0.72 gL^{-1} ethanol after 28 h incubation. The strain RPRT90 utilized 50.47± 0.69 gL^{-1} sugar during ethanol production while 8.14 ± 1.03 $gL^{-1}$was left unutilized. The unutilized sugar mainly contained pentose sugars (5.26 ± 0.71 $gL^{-1)}$ and a small amount of glucose (2.88±0.07 gL^{-1}).

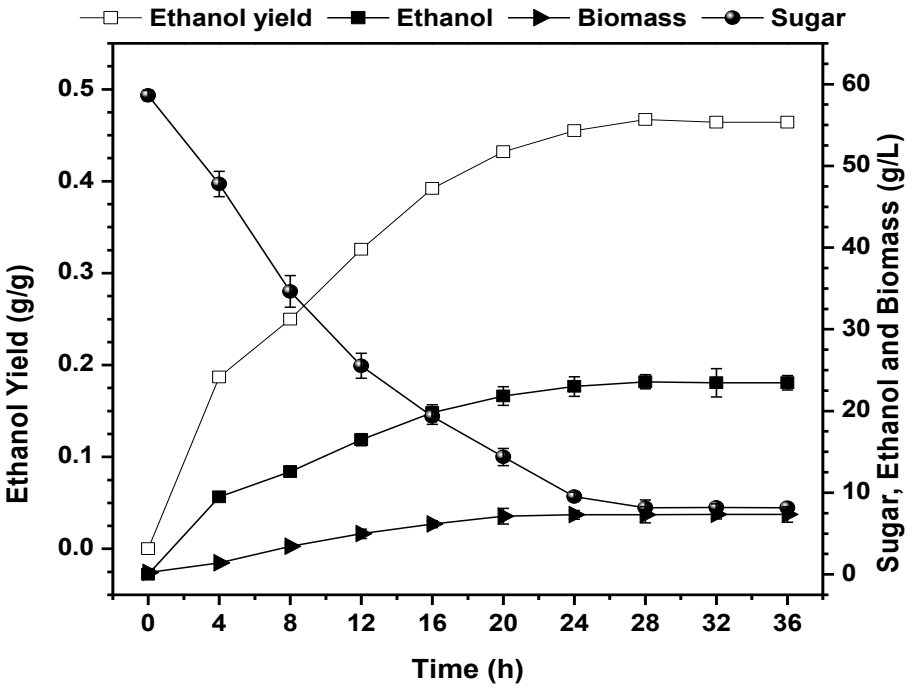


Fig. 55: Ethanol fermentation profile of mixture of detoxified acid hydrolysate and enzymatic hydrolysate of *L. camara* by fusant RPRT90

After 28 h of fermentation, the maximum ethanol productivity of 0.842 $gL^{-1}h^{-1}$, ethanol yield of 0.434 gg^{-1} and sugar conversion of 86.1%, theoretical yield of 85 % were

obtained. Thereafter, a decline in ethanol production was observed which may be because of consumption of accumulated ethanol by the yeast. The biomass increased with the increase in time and reached to a maximum of 7.33 gL^{-1} after 28 h of fermentation which remained constant till the end of fermentation.

Fermentation of mixture of undetoxified acid hydrolysate and enzymatic hydrolysate

The fermentation profile of the fermentation of mixture of undetoxified acid and enzymatic hydrolysates of *L. camara* is shown in Fig 56. The undetoxified acid hydrolysate containing 20.96 ± 0.38 gL^{-1} sugar was mixed with equal volume (2L) of enzymatically hydrolysed cellulosic hydrolysate (39.49±1.27 gL^{-1}sugars). The fermentation of the mixed hydrolysates produced 20.12 ± 0.68 gL^{-1} ethanol after 32 h incubation. The total sugar content of the mixed hydrolysate 60.45 ±0.63 gL^{-1} of sugar, 48.42 ± 0.43 gL^{-1} was consumed giving % sugar conversion of 80% while, 12.03 ±1.54 gL^{-1} was left unutilized which contained 3.64 ±0.53 gL^{-1} of glucose and 8.39 ±0.26 $gL^{-1}$of pentose sugars. The maximum ethanol productivity, ethanol yield and theoretical yield calculated after 32 h of fermentation were 0.628 $gL^{-1}h^{-1}$, 0.412 gg^{-1} and 81.3 % respectively. Thereafter, the ethanol production was observed to be constant and no significant improvement in the ethanol production was observed with increase in fermentation time. The biomass was observed to increase with time up to 28 h of fermentation, reached up to a maximum of 6.27 gL^{-1} and became constant till the end of fermentation.

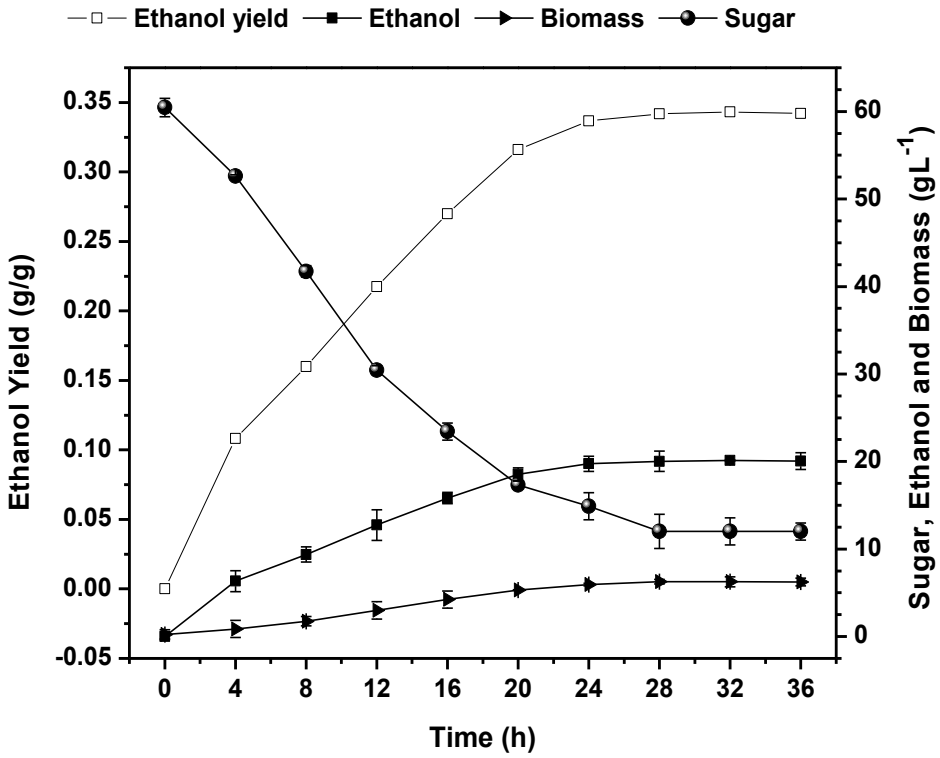


Fig 56: Ethanol fermentation profile of mixture of undetoxified acid hydrolysate and enzymatic hydrolysate of *L. camara* by fusant RPRT90

5.3.2.6 Comparison of results with published literature

The fermentation of mixed hydrolysate of *L. camara* biomass using mutant hybrid yeast CP11 was also reported by Pasha et al. The hybrid CP11 gave an ethanol yield of 0.431 ± 0.012 gg^{-1} and productivity of 0.67 ± 0.015 $gL^{-1}h^{-1}$. The yield (0.434 gg^{-1}) obtained in the present study using hybrid yeast RPRT90 is higher than CP11 [59]. In another study, the separate fermentation of acid and enzymatic hydrolysate of *L. camara* biomass using *Pichia stipitis* and *Saccharomyces cerevisiae* was performed by Kuhad et al. [67]. They reported an ethanol production of 22.46 gL^{-1} which is also considerably lower than the ethanol obtained in the present study with RPRT90 (25.45 gL^{-1}). The ethanol yields obtained from acid and enzymatic hydrolysate reported by Kuhad et al. were 0.32gg^{-1} and 0.48 gg^{-1}. Thus it has been established that RPRT90 is a potential yeast strain for bioethanol production and even has shown better activity than the activity shown by other mutant hybrid strains reported.

Chapter 6

SUMMARY AND CONCLUSION

In recent years, bioethanol has been considered as the most attractive alternative energy source for automobile industry. Due to stringent environment protection law, uncertainty in supply of petroleum based fuel and promise of bioethanol to contribute a cleaner environment, there is increasing demand of this biofuel worldwide. Though the current method of producing bioethanol using a variety of crops such as sugar cane, corn etc are well established, the exploitation of a cheaper and widely available lignocellulosic biomass can make bioethanol more competitive with petroleum based transportation fuel. However, the conversion of lignocellulosic biomass to bioethanol is much difficult due to complexity of its characteristics and its efficient conversion depends on the utilization of both pentose and hexose sugars present in this biomass. In this context, the development of potential yeast strain with desired fermentation characteristics including ability to co-ferment hexoses and pentoses, multiple stress tolerance such as high temperature, high ethanol, fermentation inhibitors and stability is of paramount importance to convert lignocellulosic biomass to ethanol efficiently.

Therefore, the main aim of the present investigation was to develop potential hybrid yeast strains that can co-ferment pentose and hexose sugar components and evaluate their efficiency towards bioethanol production from a variety of lignocellulosic biomass. The most interesting and encouraging results achieved through this research work are summarized as follows:

I. In the first phase of this dissertation work, different hybrid yeast strains were developed by the fusion of protoplasts released from the most industrially important glucose fermenting yeast, *Saccharomyces cerevisiae* and three most potential xylose fermenting yeasts such as *Pachysolen tannophilus, Candida shehatae and Pichia stipitis*. The effects of the key parameters on protoplast formation and fusion were investigated and optimum conditions were established. The maximum protoplast yield of 95.7, 83%, 89.1 and 86.5% were achieved with *S. cerevisiae, P. stipitis, P. tannophilus and C. shehatae* respectively at 3.0 mg/mL enzyme concentration and 3 h lysis time using KCl as osmotic stabilizers.

   The three different combinations of fusants such as Sc+ Ps, Sc + Cs and Sc + Pt were obtained. Under optimum conditions of pH 7.5, 35% (v/v) PEG concentration and 20 min fusion time, the fusion frequency of the protoplast was found to be in the range

65-80%, the maximum frequency of 80% being observed with the fusion of protoplast of *S. cerevisiae* and *P. tannophilus*.

II. The performance of the fusants to produce bioethanol was evaluated by fermentation experiments using a mixture of glucose and xylose, which are considered as the major sugar components of lignocellulosic biomass. Among the various fusants, RPR39 comprising of *Saccharomyces cerevisiae* and *Pachysolen tannophilus* was found to be the most efficient hybrid strain producing maximum ethanol concentration of 76.8 ± 0.31 gL^{-1}, ethanol productivity of 1.06 $gL^{-1}h^{-1}$, sugar conversion of 83.7 % and ethanol yield of 0.458 gg^{-1} at the fermentation condition of 30^{o}C, pH 4.5 and 150 rpm.

The ethanol yield achieved with RPR39 is also high compared to most of the other fusant yeast strains used for ethanol fermentation of glucose–xylose mixture reported in published literature. The fusants were sorted by adopting a rapid method using FACS and the fusant nature of RPR39 was confirmed using genetic characterization techniques such as RAPD and DNS sequencing. The sequence of the ITS1 and ITS2 and the 5.8S gene of the fusant RPR39 was submitted to Genbank with an **ACCESSION NO. JN887370**.

III. In this phase of research work, an attempt has been made for further improvement of the industrially important characteristics such as ethanol tolerance, thermotolerance, inhibitor tolerance and stability of the developed hybrid strain RPR39 by sequential mutagenesis using EMS, MNNG, near and far UV radiations as mutagens. Among the various mutants obtained, mutant RPRT90 developed by MNNNG and near UV treatment was found to be very efficient strain in terms of both substrate utilization and ethanol fermentation even under various stress conditions.

Under normal fermentation conditions the mutant produced 73.6 gL^{-1} ethanol glucose-xylose mixture with an ethanol yield, productivity and sugar conversion as 0.461 gg^{-1}, 1.05 $gL^{-1}h^{-1}$ and 86.2 % respectively. Further, under the combined effect of thermal (39^{o}C) and inhibitor stress (0.25 gL^{-1} vanillin, 0.5 gL^{-1} furfural, 4 gL^{-1} acetic acid), the mutant produced ethanol with a yield of 0.379gg^{-1}, while under combined effect of ethanol (5% v/v) and inhibitor stress the ethanol yield obtained

was 0.431 gg^{-1}. Under the synergistic effect of sugar (250 gL^{-1}), thermal (39^{o}C), ethanol (5% v/v) and inhibitor stress, the strain still shown to have good ethanol producing activity achieving an ethanol yield and productivity of 0.3 gg^{-1} and 0.59 $gL^{-1}h^{-1}$. The mutant has also shown better ethanol fermentation ability compared to fusant RPR39, parental strains and other reported mutant strains. The ethanol yield achieved by RPRT90 is also comparable to the yield (0.46 gg^{-1}) obtained with the most efficient recombinant yeast strain *S. cerevisiae* 1400 (pLNH333) reported so far under fermentative condition. The mutant RPRT90 is also found to be stable which is evident from the stability study. Therefore, it has been established that multiple induced mutation is effective in improving the stress tolerance and stability of hybrid yeast strain RPR39 and the mutant produced RPRT90 has facilitated the ethanol fermentation of glucose–xylose mixture under various stress condition. Finally, RPRT90 was characterized by RAPD and DNA sequencing techniques. The sequence of the ITS1 and ITS2 and the 5.8S gene of the fusant RPRT90 was submitted to Genbank with an **ACCESSION NO. JN887371**.

IV. In the previous section of research work, it has been demonstrated that the mutant RPRT90 is an efficient hybrid yeast strain that is capable to co-ferment glucose and xylose as model lignocellulosic substrates and hence further study was undertaken to investigate the effectiveness of this strain towards bioethanol production from an unexploited and locally grown weed biomass, *Ipomoea carnea*. From the biomass composition analysis, the *I. carnea* was found to contain 49.6 ± 2.05% cellulose, 16.4 ± 0.97% hemicelluloses and 24.8 ± 0.74% lignin. The high carbohydrate content (66.5%) of *I. carnea* biomass makes it a potential source for bioethanol production.

So, a systematic research effort has been given in this phase of work for the exploitation of *I. carnea* biomass for bioethanol production through the optimization of various steps involved in its conversion such as dil. acid pretreatment for the removal of lignin and release of cellulose fibers, enzymatic hydrolysis to convert cellulose to glucose and fermentation of hexose and pentose sugars of hydrolysates to ethanol using RPRT90.

V. *I. carnea* biomass was pretreated with different acids such as sulphuric, hydrochloric and phosphoric acid. Among these, sulphuric acid was found to be the

most efficient pretreatment reagent. The influence of key pretreatment parameters using dil. sulphuric acid was studied and under optimum conditions of 3% H_2SO_4, 120^oC, 45 min pretreatment time, 21.03 gL^{-1} sugar was produced along with the formation of 1.052 gL^{-1} phenolics and 1.92 gL^{-1} furans as toxic inhibitors.

The acid pretreated biomass was further detoxified to remove inhibitory by-products using overliming and activated charcoal adsorption methods individually and in combination. The sequential treatment of overliming and activated charcoal was found to be the most favourable resulting in 91.9% furans and 94.7% phenolics removal from acid hydrolysate. Finally, the delignification of the pretreated biomass was optimized and a maximum lignin removal of 75.6% was achieved at 140 ^oC, 45 min using 20%, w/v sodium sulphite as delignifying agent.

VI. The study on the optimization of enzymatic hydrolysis of the delignified biomass by response surface methodology using cellulase and β-glucosidase enzyme resulted a saccharification yield of 746.8 mg/g at optimum condition of 50^oC, 2.5 FPU/mL cellulase concentration, 0.75 % Tween 80 supplemented with 0.25% BSA and 7.5% biomass loading.

VII. The batch fermentation experiment was carried out in a 5L bench top fermenter with the mixture of equal volume of acid and enzymatic hydrolysates using hybrid yeast strain RPRT90. Fermentation of mixed hydrolysate at 30^oC for 28 h produced 27.2 gL^{-1} ethanol with 0.456 gg^{-1} ethanol yield, 89.2% sugar conversion and 0.971 $gL^{-1}h^{-1}$ productivity.

Furthermore, since the strain RPRT90 has been demonstrated to be inhibitor tolerant, the efficiency of this strain was assessed towards fermentation of undetoxified acid hydrolysate. The fermentation of the mixture of undetoxified acid hydrolysate and enzymatic hydrolysate achieved ethanol yield and productivity of 0.415 gg^{-1} and 0.821 $gL^{-1}h^{-1}$. The result shows that the difference between the ethanol production using detoxified and undetoxified hydrolysate is quite marginal. Therefore, by the use of RPRT90 it may be possible to avoid detoxification as an

additional pretreatment step that will ultimately reduce the cost of bioethanol production.

VIII. Finally, an attempt has been made for the exploitation of the developed hybrid strain RPRT90 towards bioethanol production from another weed biomass *L. camara* which has been previously considered as a potential feedstock for bioethanol production. The experimental results were found to be similar to the results obtained with *I. carnea*. Overall, 86.1% sugar conversion, 0.842$gL^{-1}h^{-1}$ ethanol productivity, 0.434gg^{-1} ethanol yield, and 85% theoretical yield were obtained after 28 h of fermentation of *L. camara* mixed hydrolysate containing enzymatic and detoxified acid hydrolysates. Furthermore, the fermentation of the mixture of enzymatic and undetoxified acid hydrolysate resulted in ethanol productivity, ethanol yield and theoretical yield of 0.628 $gL^{-1}h^{-1}$, 0.412 gg^{-1} and 81.3 % respectively. The strain has thus shown good catalytic activity in converting mixed hydrolysate containing undetoxified and detoxified hydrolysates.

IX. A comparison between ethanol productions from both weed biomass used in this study shows that the ethanol produced by *I. carnea* is slightly higher than *L. camara*. The high amount of ethanol may be due to the higher carbohydrate content of *I. carnea* biomass compared to *L. camara*.

Overall, in the present investigation, a genetically stable and potent glucose- xylose fermenting hybrid yeast strain RPRT90 was developed by protoplast fusion followed by sequential mutagenesis. The strain was found to be very efficient in fermenting the major sugar components (glucose and xylose sugars) of lignocellulosic biomass even under multiple stress fermentation condition. Furthermore, through this present study, it is the first time *I. carnea* has been found to be a potential feedstock for bioethanol production. The developed strain RPRT90 has been successfully employed for the production of bioethanol from *I. carnea* and *L. camara*. It is finally concluded that the development of ethanol fermentation system using RPRT90 may pave the way for large scale production of bioethanol from a variety of lignocellulosic biomass efficiently even with the elimination of detoxification step that will further offer the bioethanol production in a cost effective manner.

Chapter 7

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LIST OF PUBLICATIONS

Journal Publications

Papers published

1. Rajni Kumari, Krishna Pramanik: Improvement of stress tolerance in yeast strain by sequential mutagenesis for enhanced bioethanol production, Journal of Bioscience and Bioengineering, (in press), DOI: 10.1016/j.jbiosc.2012.07.007 (Elsevier Publications).

2. Rajni Kumari, Krishna Pramanik: Improved bioethanol production using fusants of *Saccharomyces cerevisiae* and xylose-fermenting yeasts, Applied Biochemistry and Biotechnology, Vol. 186, No. 4, 873-884 (2012) (Springer Publications).

3. Rajni Kumari, Navneet Kumar Dubey, Krishna Pramanik: Bioethanol: The liquid gold of the tomorrow from lignocellulosic wastes, Journal of Environmental Research and Development (JERAD), Vol. 3, No.-3, pp. 922-927 (2009) (JERAD Publications).

Papers communicated

4. Rajni Kumari, Krishna Pramanik: Bioethanol production from *Ipomoea carnea* using an inhibitor tolerant yeast mutant" communicated to Biomass and Bioenergy, Elsevier.

5. Rajni Kumari, Krishna Pramanik: Bioethanol production from *Lantana camara* using an inhibitor tolerant yeast mutant" communicated to Biosystems Engineering, Elsevier.

Papers under preparation

6. Rajni Kumari, Krishna Pramanik: Kinetics of ethanol production from glucose-xylose mixture using a hybrid yeast strain.

Book Chapter

1. Rajni Kumari, Navneet Kumar Dubey, Krishna Pramanik: Development of genetically modified microbial strains for enhanced bioethanol production from agricultural wastes, Book chapter in "Socio-Economic Development-Challenges before Women Scientists, Technologists & Engineers", 2009, (ISBN #978-93-80043-03-6). pp. 109-114 (2008).

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